REMARKS

The Amendments

Claim 1 is amended to incorporate the substance of previous claim 6 (except to exclude "ariflo" (= cilomilast)) and recite additional components of the composition; see, e.g., page 17, lines 10-25. The claims are otherwise amended to conform with these amendments or make non-substantive clarifications. Claims 1-2, 4-5, 7-11, 13, 19-38 and 43-44 remain pending herein.

To the extent that the amendments avoid the prior art or for other reasons related to patentability, competitors are warned that the amendments are not intended to and do not limit the scope of equivalents which may be asserted on subject matter outside the literal scope of any patented claims but not anticipated or rendered obvious by the prior art or otherwise unpatentable to applicants. Applicants reserve the right to file one or more continuing and/or divisional applications directed to any subject matter disclosed in the application which has been canceled by any of the above amendments.

The Rejection under 35 U.S.C. §112, second paragraph

The rejection of claims 6 and 7 under 35 U.S.C. §112, second paragraph, is respectfully traversed.

The identifiers of the specific PDE-IV inhibitors previously recited in claim 6, which were objected to, now appear in claim 1. Applicants respectfully submit that these terms (Bay-198004, CP-325,366, BY343, D-4396 (Sch-351591), V-11294A, AWD-12-281) identify specific compounds which would have been known to one of ordinary skill in the art. As proof of the knowledge in the art of these terms identifying specific compounds, attached are literature excerpts associating each of the identifiers with a specific compound (see

Attachment A). The excerpts also make clear that those skilled in the art identified these terms with specific compounds. Since the claims identify that these compounds are PDE-IV inhibitors, it would be clear to those skilled in the art that the terms can only refer to the particular PDE-IV inhibitors known in the art under such identifiers, such as shown by the excerpts. Since one of ordinary skill in the art would understand the meaning and scope of the claims when read in light of the specification, they are not indefinite under 35 U.S.C. §112, second paragraph; see, e.g., Morton Int. Inc. v. Cardinal Chem. Co., 28 USPQ 1190 (Fed. Cir. 1993).

The objection to the "ariflo" trademark term is rendered moot since it no longer appears in the claims.

For the above reasons, the rejection under 35 U.S.C. §112, second paragraph, should be withdrawn.

The Rejection under 35 U.S.C. §112, first paragraph

The rejection of claims 1-5 and 8-43 under 35 U.S.C. §112, first paragraph, is believed to be rendered moot by the above claim amendments. The rejection was not applied to claim 6 and the substance of claim 6 relevant to this rejection is now incorporated into independent claim 1, upon which all other claims ultimately depend. The rejection was based on the allegation that the disclosure was not enabling for the unspecified "PDE-IV inhibitors" term. The claims now recite specific PDE-IV inhibitors, which the Office Action acknowledges are enabled. Thus, the rejection should be withdrawn.

The Rejection under 35 U.S.C. §103

The rejection of claims 1-43 under 35 U.S.C. §103, as being obvious over Knowles (WO 03/011274) in view of Meissner (U.S. Patent No. 6,706,726) further in view of Hill

(U.S. Patent No. 6,060069) is respectfully traversed.

Knowles discloses, generally, inhalable compositions containing a PDE-4 inhibitor and an anticholinergic agent; see, e.g., page 1, lines 1-7. As the PDE-4 inhibitor, Knowles particularly directs one of ordinary skill in the art towards those having a selective activity, specifically cilomilast or Ariflo®; see, e.g., page 3, line 25, through page 4, line 27. As anticholinergic agents, Knowles refers to such agents which show particular receptor antagonist activity and specifies atropine, scopolamine, homatropine, hyoscyamine and related compounds; see, e.g., page 4, line 28, to page 5, line 31. The only particular examples of compositions disclosed by Knowles combine cilomilast (PDE-4 inhibitor) with tiotropium (anticholinergic).

As acknowledged in the Office Action, Knowles does not disclose or suggest use of an anticholinergic according to formula **1** of the instant claims. Knowles provides no suggestion to modify the anticholinergics taught therein in a manner which would suggest a compound of formula **1** of the instant claims. Applicants further submit that Knowles is distinguished from the instant claims because it fails to give specific direction to a composition containing one of the specific PDE-4 inhibitors now recited in claim 1. Note that the instant claims do not recite cilomilast (=Ariflo®) as a PDE-4 inhibitor.

Meissner discloses compounds of its formula 1 as anticholinergics which formula encompasses formula **1** of the instant claims. It is alleged in the Office Action that it would have been obvious to one of ordinary skill in the art to modify the Knowles compositions to use an anticholinergic according to Meissner and combine that with a PDE-4 inhibitor. The alleged basis of motivation for doing so is that Meissner and Knowles compounds are structurally similar and would be expected to have similar properties.

Applicants respectfully disagree that the Meissner compounds and, particularly, the compound of instant claim **1**, are structurally similar to the anticholinergics disclosed by

Knowles. The structures of the 4 anticholinergics specifically taught by Knowles are shown in an attachment to this Reply (see Attachment B). It can be seen that these compounds lack a significant structural feature of Meissner's general formula 1. That is, these compounds have only one benzene ring where the Meissner compounds have two benzene rings on a single carbon. Instead, the Knowles compounds have an additional $-CH_2OH$ group which is not present in the Meissner compounds. Further, as to the specific compound of Meissner's Example 1 (also formula **1** of the instant claims), the Knowles compounds are additional distinct for: lacking an additional methyl group on the carbon which has the two benzene rings, lacking an additional methyl substituent on the nitrogen in the polycyclic ring which creates an ammonium group and provides for the anion, and only the scopolamine compound have the epoxy group fused to the polycyclic ring. Thus, there are several significant structural differences.

In view of the significant structural differences, one of ordinary skill in the art would not have had a reasonable expectation that the Meissner compounds, particularly of Example 1, could be substituted in the Knowles compositions with the expectation of similar properties and results.

In addition, Meissner itself supports that the compounds would not be expected to exhibit the same properties or results. Meissner refers to compounds of the type disclosed as anticholinergics in Knowles in its Background section (col. 1, line 33, to col. 2, line 26) and discloses that such compounds are deficient in meeting the requirements desired for the Meissner invention. Thus, Meissner's invention is directed to its structurally distinct compounds with distinct properties. Meissner's disclosure, thus, further supports that one of ordinary skill in the art would not have had a reasonable expectation that the Meissner compounds would have similar properties to the anticholinergics of Knowles and could be substituted into the Knowles compositions with a reasonable expectation of success.

Additionally, Knowles teaches that a specific type of anticholinergic is desired which has a certain M_1 and M_2 receptor antagonist activity; see paragraph bridging pages 4-5 of Knowles. Meissner provides no disclosure either way as to whether its compounds possess such antagonist activity. Thus, whether or not the compounds actually possess such activity, the failure of Meissner to teach such activity further detracts from any reasonable expectation of success by one of ordinary skill in the art in substituting the Meissner compounds, particularly selecting the specific compound of Example 1, into the Knowles compositions.

Finally, Meissner does not suggest the use of its anticholinergics of formula 1 together with PDE-4 inhibitors. This also detracts from any reasonable expectation by one of ordinary skill in the art that the Meissner compounds would be useful in compositions with PDE-4 inhibitors.

For all of the above reasons, it is urged that the combined teachings of Knowles and Meissner fail to suggest the claimed invention to one of ordinary skill in the art.

Hill was relied upon in the Office Action for suggesting certain dependent claim embodiments regarding particular excipients in the compositions. Hill provides no suggestions to make up for the deficiencies of the combination of Knowles and Meissner discussed above. Hill provides no suggestion of combining a compound of Meissner's formula 1 as an anticholinergic in the PDE-4 inhibitor compositions of Knowles. Therefore, while applicants reserve the right to discuss the distinction of the combined teachings of Hill for such dependent claim features, such discussion is not believed to be necessary at this time to establish nonobviousness.

For all of the above reasons, it is respectfully submitted that the combined teachings of Knowles, Meissner and Hill fail to suggest the claimed invention to one of ordinary skill in the art. Thus, the rejection under 35 U.S.C. §103 should be withdrawn.

The Provisional Obviousness-type Double Patenting Rejections

The provisional obviousness-type double patenting rejection of claims 1-7 and 43 over claims 1-13 of US Ser. No. 10/613,783 in view of claims 1-8, 11 and 21-23 of U.S. Patent No. 6,706,726 is respectfully traversed.

Because this is a provisional application and both this application and the copending application are currently subject to other grounds of rejection, positive action, such as by filing a terminal disclaimer, to overcome this rejection would be premature. For example, the claims in either application could be amended in a manner to remove the rejection. Thus, any such positive action is held in abeyance.

However, in analogy to the traversal of the 35 U.S.C. §103 rejection above, applicants urge that the instant claims are not an obvious variant of the claims of the copending '783 application. The anticholinergics disclosed for use in the '783 application are similar to those disclosed by Knowles. The basis alleged in the Office Action for combining the teachings of Meissner, and alleging that compositions substituting Meissner's anticholinergics in the '783 compositions would be an obvious variant of the '783 compositions, is that Meissner's compounds of the compound of formula **1** of the instant claims are allegedly of similar structure and use to the anticholinergics taught by Knowles. Thus, the reasoning to support the rejection is essentially the same as for the 35 U.S.C. §103 rejection. As a result, essentially the same traversal made above applies. To summarize, the Meissner compounds of formula 1, and particularly the compound of formula **1** of the instant claims, are not so structurally similar to the anticholinergics taught in '783 such that one of ordinary skill in the art would expect them to possess similar properties. Further evidence in the prior art, i.e., Meissner and Knowles as discussed above, detracts from one of ordinary skill in the art having a reasonable expectation that the Meissner compounds could be substituted in the '783

compositions to achieve the same or similar properties or results. Accordingly, applicants urge that the instant claims are not obvious variants of the '783 claims in view of the prior art. Thus, the provisional obviousness-type double patenting rejection should be withdrawn.

It is submitted that the claims are in condition for allowance. However, the Examiner is kindly invited to contact the undersigned to discuss any unresolved matters.

Respectfully submitted,

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ATTACHMENT A

BAY 19-8004-1

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Phosphodiesterase 4 Inhibitors for the Treatment of COPD*

Graham Sturton and Mary Fitzgerald

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Phosphodiesterase 4 Inhibitors for the Treatment of COPD*

Graham Sturton, PhD; and Mary Fitzgerald, PhD

Phosphodiesterase 4 (PDE4) is a major cyclic adenosine-3′,5′-monophosphate-metabolizing enzyme in immune and inflammatory cells, airway smooth muscle, and pulmonary nerves. Selective inhibitors of this enzyme have been available for a number of years and show a broad spectrum of activity in animal models of COPD and asthma. The class-associated side effects, mainly nausea and emesis, appear to have been at least partially overcome by the so-called "second-generation" PDE4 inhibitors. Currently, three companies are in the later stages of development of candidate second-generation PDE4 inhibitors for the treatment of COPD patients. The preclinical profile of one of these, BAY 19–8004, is summarized below. The initial clinical data on the most advanced compound, cilomilast, were indeed encouraging. However, full knowledge of the therapeutic value of this novel compound class awaits the outcome of longer term clinical trials.

(CHEST 2002; 121:192S–196S)

Key words: BAY 19–8004; cilomilast; COPD; inhibitor; phosphodiesterase; roflumilast; tobacco smoke

Abbreviations: Cmax = peak plasma concentration; ED_{50} = median dose for 50% inhibition; LPS = lipopolysaccharide; PDE4 = phosphodiesterase 4

The defining feature of COPD is an accelerated decline in lung function that is, in the overwhelming majority of cases, caused by cigarette smoking and is largely irreversible. Like asthma, COPD is associated with airway inflammation.[1] However, whereas the inflammatory process in asthma can be described simplistically as a CD4+ T-cell-driven eosinophilia, COPD is marked by an increase in the numbers or activity of CD8+ T cells, macrophages, and neutrophils. Long-term trials[2–4] of inhaled corticosteroids in COPD patients have failed to show significant benefit in terms of slowing the progression of this disease. Furthermore, unlike asthma, important elements of this inflammatory response are insensitive to steroids,[5,6] thus highlighting the need for novel anti-inflammatory therapies.

Phosphodiesterase 4 (PDE4) inhibitors have been shown to relax airway smooth muscle, to suppress the activation of inflammatory cells, and to modulate the activity of pulmonary nerves.[7,8] The reported effects of PDE4 inhibitors *in vitro* and in animal models suggest that, in addition to short-term effects on bronchomotor tone, they may find utility in reducing the protease burden associated with neutrophilic inflammation, as well as down-regulating the activity of CD8+ T cells and macrophages. Such effects have the potential to slow the accelerated decline in lung function seen in patients with COPD and, thus, to modify the natural history of this disease.

Despite the wealth of publications documenting the broad anti-inflammatory profile of PDE4 inhibitors *in vitro*, it is worth noting certain limitations that may prove to be of importance in defining their anti-inflammatory impact in the clinical setting. It is clear that some inflammatory cells are less sensitive to PDE4 inhibition than others. Within the same cell type, different functions display different sensitivities to cyclic adenosine-3′,5′-monophosphate elevation. In the neutrophil, degranulation is relatively insensitive compared to superoxide production and leukotriene B_4 generation. Thus, some anti-inflammatory effects reported *in vitro* may not be expressed at plasma levels that are attained in humans. A further feature of their profile of activity on neutrophils is that elevation of cyclic adenosine-3′,5′-monophosphate, at least in normal circulating cells, results in delayed apoptosis,[9,10] which could have proinflammatory consequences.

At present, three companies have compounds that are in the late stages of development for COPD treatment (Fig 1). The furthest advanced compound is cilomilast (Ariflo [SB207499]; GlaxoSmithKline; Uxbridge, UK). In a 6-week phase II study[11,12] in patients with moderate COPD, cilomilast caused significant improvements in both lung function and symptom scores at a dose of 15 mg bid. Roflumilast (Byk Gulden; Konstanz, Germany) appears to be the most potent of the three compounds with a dose of 0.5 mg once daily being assessed in phase II. Efficacy has been reported in a phase II study of asthmatic patients,[13] but phase II results for both roflumilast and BAY 19–8004 (Bayer AG; Wuppertal, Germany) in COPD patients have not yet been published.

Preclinical Profile of BAY 19–8004

In Vitro

BAY 19–8004 is representative of a new structural class of PDE4 inhibitors, the benzofurans. The profile of BAY 19–8004 *in vitro* is summarized in Table 1. In common with cilomilast and roflumilast, it is highly selective for PDE4. The mean concentration required for 50% inhibition of PDE4 that was present in a membrane preparation from human neutrophils was 67 nm. As an inhibitor of other PDE1, -2, -3, and -5 isoform enzymes from a variety of sources, BAY 19–8004 showed < 50% inhibition at a concentration of 10 μM. BAY 19–8004 does not show significant selectivity for a particular subtype of PDE4.

There was a highly significant correlation between potency against this neutrophil PDE4 activity and inhibition of neutrophil superoxide generation for a range of benzofurans. BAY 19–8004 inhibited human inflammatory cell functions that previously were reported to be sensitive to PDE4 inhibitors. It showed some selectivity for neutrophil and eosinophil functions over those of monocytes and T lymphocytes when compared to cilomilast and other reference PDE4 inhibitors. However, this

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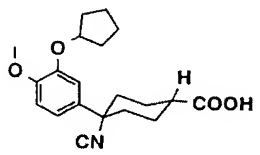
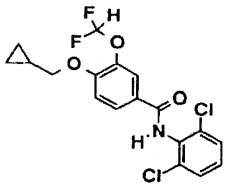
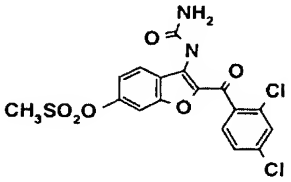
Compound	Structure	Maximum Dose (Phase II)	Status
Ariflo™ (Glaxo Smith-Kline)		15 mg b.i.d. orally	**Phase III**
Roflumilast (Byk Gulden)		0.5 mg o.d. orally	**Phase II**
BAY 19-8004 (Bayer)		5 mg o.d. orally	**Phase II**

FIGURE 1. PDE4 inhibitors in advanced clinical development for COPD.

qualitative difference was not reflected in any differences in the profile of activity between cilomilast and BAY 19–8004 in animal models.

In Vivo

As is expected from this class of compounds, BAY 19–8004 exhibited a broad profile of anti-inflammatory activity in animal models of COPD and asthma (Table 2). It was also an effective bronchodilator. When administered IV to guinea pigs that previously had received aerosol leukotriene D_4 to induce a sustained bronchoconstriction, BAY 19–8004 significantly reversed the leukotriene D_4-induced response with a median for 50% inhibition dose (ED_{50}) of 0.3 to 1 mg/kg. While in standard guinea pig antigen models the ED_{50} of the compound was in the range of 1 to 3 mg/kg, in rat and cynomolgus monkey models the potency was higher, and the associated maximum plasma levels (*ie*, maximum plasma concentration) at the ED_{50} were 60 and 29 ng/mL, respectively.

Table 1—*Molecular and Cell Function Profile of BAY 19–8004**

Test	Stimulus	BAY 19–8004 IC_{50}, nM
Human neutrophil PDE4	NA	67
[^{3}H]-Rolipram binding (rat brain)	NA	14
Human neutrophil O_2	fMLP	38
Human neutrophil leukotriene B_4	fMLP	2
Human eosinophil leukotriene C_4	fMLP	0.02
Human monocyte TNF-α	LPS	260
Human lymphocyte interleukin-5	PHA	670

*fMLP = formyl-methyl-leucyl-phenylalanine; PHA = phytohemagglutinin; IC_{50} = mean concentration required for 50% inhibition; NA = not applicable; TNF = tumor necrosis factor.

The effects of earlier PDE4 inhibitors have been well-documented in the aforementioned models. A feature of COPD that has been less well-studied in relation to PDE4 inhibition is the hypersecretion of mucin. We used a guinea-pig model in which the instillation of bacterial lipopolysaccharide (LPS) results in a sixfold increase in the output of immunoreactive mucin. Pretreatment with BAY 19–8004 (10 mg/kg po) resulted in the complete inhibition of LPS-induced mucin hypersecretion. Inhibition (46%) also was observed with cilomilast at 30 mg/kg po, although this was not statistically significant. Interestingly, the influx of neutrophils in response to LPS was not significantly inhibited in these animals by either PDE4 inhibitor. The inhibition of LPS-induced neutrophil influx in the guinea pig has been reported for cilomilast.[14] The failure to observe this in the present experiments may be due to the higher instillation dose of LPS that is required

Table 2—*Profile of BAY 19–8004 in Animal Models of COPD and Asthma**

Test	Dose	Plasma Levels, Cmax	Effect
Guinea-pig			
Antigen-induced bronchoconstriction	3 mg/kg po	ND	60% inhibition
Antigen-induced eosinophilia	3 mg/kg po	ND	63% inhibition
Allergic cynomologus monkey	0.1 mg/kg/d po	29 ng/mL	65% inhibition of eosinophil influx; 100% inhibition hyperresponsiveness
LPS			
Rat	1 mg/kg po	100 ng/mL	62% inhibition of neutrophil influx
Rat	10 mg/kg po	ND	91% inhibition of TNF-α; 89% inhibition of MIP-α
Guinea pig	10 mg/kg po	ND	100% inhibition of mucin output
Tobacco smoke-induced inflammation in guinea pigs	10 mg/kg po	ND	100% inhibition of increase in inflammatory cells in BAL fluid

*ND = not determined; MIP-α = monocyte inhibition protein-α. See Table 1 for other abbreviation.

to elicit mucin hypersecretion. It does, however, suggest that PDE inhibitors could inhibit mucin hypersecretion directly rather than by an indirect mechanism involving neutrophils.

There is currently no information reporting the evaluation of PDE4 inhibition in models of tobacco smoke-induced inflammation and emphysema. In collaboration with Dr. James Hogg (St. Paul Hospital; Vancouver, BC, Canada), we have investigated the effects of BAY 19–8004 in such a model. There is a significant inflammatory response in the lungs of guinea pigs 1 h after exposure to tobacco smoke (*ie*, five cigarettes). The levels of neutrophils, macrophages, and eosinophils all were increased in the BAL fluid. The neutrophil component of this inflammatory response was not inhibited at a dose of 5 mg/kg betamethasone. By contrast, BAY 19–8004 (10 mg/kg po) completely inhibited the influx of all inflammatory cell types. Studies are currently ongoing to determine the effects of BAY 19–8004 on both the inflammatory and emphysematous response that develops after 12 weeks of tobacco smoke exposure.[15] In all efficacy models in which comparisons were performed, BAY 19–8004 was 10- to 30-fold more potent than cilomilast.

Class-Associated Side Effects

The promise that PDE4 inhibitors will have an improved side-effect profile over nonselective compounds has been borne out in early clinical trials, at least with regard to cardiovascular and most CNS side effects. However, GI side effects, including nausea, vomiting, and dyspepsia, limit the dosages of these compounds that can be administered to humans.[16–18]

The long splice variants of PDE4 can exist as two conformers. One with a high affinity for rolipram predominates in parietal cells and CNS tissue. A second conformer with low affinity for rolipram is present in inflammatory cells. Most, though not all, anti-inflammatory effects are mediated by this form.[19] Reducing activity in the high-affinity form while improving inhibitory potency in the low-affinity form has been suggested as a strategy for improving the therapeutic window of PDE4 inhibitors.[19]

When compared to the archetypal PDE4 inhibitor rolipram, cilomilast shows such an improved relative potency on these two conformers. Nevertheless, at the clinically effective dose (15 mg bid), nausea and headache still were reported, although they were transient in nature. In phase 1 studies, the higher dose of 20 mg bid was reportedly not tolerated.[17]

BAY 19–8004 shows similar relative activities to cilomilast on the two PDE4 conformers (Table 1). Despite the lack of emesis in a ferret model, BAY 19–8004 produced emesis in primates. The threshold dose of BAY 19–8004 for this effect in primates was 10-fold lower than that for cilomilast, suggesting a similar therapeutic window in animal models. The slow absorption of BAY 19–8004 in humans may have an additional benefit with respect to its side-effect profile.

Summary of Phase 1 Findings With BAY 19–8004

BAY 19–8004 exhibited linear pharmacokinetics with a half-life of 25 h and low plasma clearance in phase 1 studies. There was low intersubject variability. A once-daily administration of 5 mg (the highest dose subsequently used in phase II COPD studies) to elderly patients achieved plasma levels in the range associated with efficacy in animal models (maximum plasma concentration, 68 ng/mL; minimum plasma concentration, 40 ng/mL at steady state). Once-daily administration is therefore the dosing regimen envisaged.

There were no relevant findings with regard to circulation, lung, liver, kidney, or hematology. The most frequently reported adverse event was nausea. The incidence of nausea was dose-related, and, as was reported for cilomilast, it occurred early in the dosing regimen and was transient in nature for most subjects.

To date, there have been no reports regarding the activity of PDE4 inhibitors against features of lung inflammation in either asthmatic or COPD subjects. However, in a recent phase II asthma study, roflumilast provided statistically significant (21%) inhibition of LPS-induced tumor necrosis factor-α production from whole blood *ex*

vivo.[20] In phase I studies, we exploited a property of BAY 19–8004 that had not been exhibited by the other PDE4 inhibitors so far examined. The inhibition of superoxide production persists in leukocytes isolated from blood treated with BAY 19–8004. This is despite the fact that this isolation involves at least two hypotonic lysis steps and facilitates the monitoring of systemic anti-inflammatory activity of the compound. A statistically significant (*ie*, up to 46%) dose-related inhibition of leukocyte superoxide production *ex vivo* was observed in phase I volunteers treated with doses up to 15 mg BAY 19–8004 per day.

What Will Third-Generation PDE4 Inhibitors Look Like?

From the available information on cilomilast, it appears that while the side effects are apparently tolerated at a dose showing efficacy (*ie*, 15 mg bid), they are still a significant problem and almost certainly limit the dose.

Two strategies for further improvement in the therapeutic window of PDE4 inhibitors can be envisaged. The finding that PDE4 exists as four genetically distinct subtypes offers the possibility of identifying subtype-selective inhibitors. There is some evidence that such compounds can target specific inflammatory cell functions.[21,22] However, the selectivity achieved to date has been limited, and there are no published data on the association with side effects. A second approach would be to further reduce potency in the high-affinity conformer. Both cilomilast and BAY 19–8004 represent significant improvements on rolipram in this regard. A compound described in a recent patent suggests that significant further improvement is possible.[23] Whether such a compound will overcome the current dose limitation imposed by the side effects of nausea and emesis remains to be investigated.

Conclusion

The preclinical data supporting the potential utility of PDE4 inhibitors in COPD are compelling. The observed efficacy of cilomilast in COPD patients also is encouraging. However, it is not clear whether the visible effects on lung function and symptom score are a manifestation of the bronchodilator activity of PDE4 inhibitors or are a consequence of anti-inflammatory effects. Early reports suggest that significant anti-inflammatory effects were not seen. The optimal positioning of PDE4 inhibitors in the treatment of COPD patients is dependent on the demonstration of anti-inflammatory activity. This might predict a beneficial effect on the accelerated rate of lung function decline, which would be a major breakthrough in the treatment of patients with this disease.

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The Investigational Drugs database

DRUG report

BAY 19-8004-2

lirimilast

Company	**Bayer AG**
Highest Dev Status	Suspended
Indications	Chronic obstructive pulmonary disease Asthma
Actions	PDE 4 inhibitor Bronchodilator
Technologies	Tablet formulation

Reason for update on 18 April 2005

one or more development status entries have been updated, 1 reference added [**593082**]

Summary

Bayer was developing lirimilast (BAY-19-8004), a benzofuran derivative phosphodiesterase 4 (PDE4) inhibitor, for the potential treatment of asthma and chronic obstructive pulmonary disease (COPD) [**317452**], [**319225**], [**412857**]. By June 2001, lirimilast had reached phase II development for COPD and asthma; however, at that time Bayer disclosed that it would not continue the development of lirimilast for asthma. At this time, development of the drug for COPD was suspended until all phase II findings had been evaluated [**412857**]. By March 2002, it appeared that the trials had resumed [**443846**]; however, by March 2005, development activities were stopped. At that time, Bayer stated that further options to exploit the potential of the compound were under investigation [**593082**].

It was presumed that **Argenta Discovery** (formerly Etiologics), following its acquisition of Etiologics in October 2004 [**562143**], was investigating lirimilast for the potential treatment of COPD under license from Bayer. In June 2003, Etiologics acquired Bayer's UK preclinical respiratory research group [**493875**]. This deal appeared to involve lirimilast [**515457**]; however, in November 2003, both companies declined to clarify the licensing situation for the drug. No development has been reported by the company since 2003.

PRECLINICAL STUDIES

In October 2003, preclinical data on lirimilast were presented at the SMi Anti-Inflammatory Therapeutics meeting in London, UK. Lirimilast inhibited human PDE4 with an IC50 value of 67 nM [**509735**].

In May 2003, Etiologics presented data on the effects of lirimilast on pulmonary inflammation induced by tobacco smoke at the American Thoracic Society International Conference in Seattle, WA [**515457**].

According to data presented in May 2000, lirimilast exhibited potential as a stand-alone therapy in mild asthmatics, and did not exhibit the side effect profile of steroids [**397137**].

ADDITIONAL INFORMATION

In June 2003, Etiologics acquired Bayer's UK preclinical respiratory research group [**493875**]; it is presumed that lirimilast formed part of this deal [**515457**].

In February 1999, Lehman Brothers predicted a 10% probability that lirimilast would reach the market, and launch by 2005. Peak annual sales of $400 million (worldwide) were predicted for 2013 [**319225**]. In May 2000, Bayer expected peak sales of EUR 750 million and EUR 1250 million for asthma and COPD, respectively [**397137**].

Development Status

HISTORY

Detailed status for **Argenta Discovery**

Indication	Country	Status	Reference	Date
Chronic obstructive pulmonary disease	UK	No Development Reported		17 December 2004

Detailed status for **Bayer AG**

Indication	Country	Status	Reference	Date
Asthma	Germany	Discontinued	**412857**	18 June 2001
Chronic obstructive pulmonary disease	Germany	Suspended	**593082**	15 March 2005

Detailed status for **Bayer Yakuhin Ltd**

Indication	Country	Status	Reference	Date
Chronic obstructive pulmonary disease	Japan	Suspended	**593082**	15 March 2005

Licensing

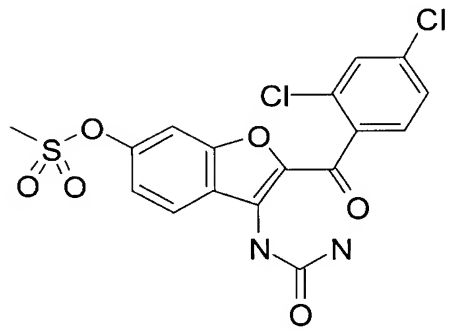
Summary — Reference

Etiologics Ltd

In June 2003, **Etiologics** acquired **Bayer**'s UK preclinical respiratory research group [493875]; it is presumed that **lirimilast** formed part of this deal [**515457**]. However, no development has been reported by Etiologics since that time. **493875**

Chemistry

Structure



Confidence Level : 1

Compound names associated with this drug

Name	Type
329306-27-6	CAS RN
BAY-19-8004	Research Code
lirimilast	INN

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The Investigational Drugs database

DRUG report

Drug Report

CP-325366

tofimilast

Company	**Pfizer Inc**
Highest Dev Status	Clinical
Indications	Allergy Respiratory disease
Actions	PDE 4 inhibitor Bronchodilator

Reason for update on 03 July 2003

chemical structure added or updated

Related information

COMPANY

REFERENCES

NEWS

Actions

EMAIL IF UPDATED

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Summary

Pfizer is developing **tofimilast** (CP-325366), a member of a series of phosphodiesterase type 4 (PDE4) inhibitors, for the potential treatment of allergy and respiratory disease [**443406**], [**494388**]. By June 2003, **tofimilast** (structure shown) was described as being in 'mid-stage' development (assumed to mean phase I or phase II clinical trials) for allergy and respiratory indications [**494388**].

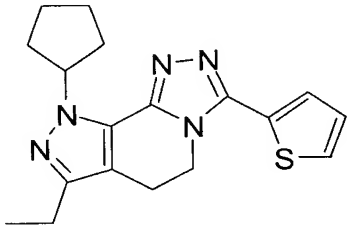
Development Status

Detailed status for **Pfizer Inc**

Indication	Country	Status	Reference	Date
Allergy	US	Clinical	**494388**	17 June 2003
Respiratory disease	US	Clinical	**494388**	17 June 2003

Chemistry

Structure



Confidence Level : 1

Compound names associated with this drug

Name	Type
185954-27-2	CAS RN

478409-36-8	CAS RN
CP-325366	Research Code
tofimilast	USAN, INN

Literature evaluation

As of July 2003, there were insufficient data available on which to base an evaluation of this drug.

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The Investigational Drugs database
DRUG report

BY 343

pumafentrine	
Company	**ALTANA Pharma AG**
Highest Dev Status	Discontinued
Indications	Chronic obstructive pulmonary disease Asthma Bronchitis
Actions	Anti-inflammatory PDE 3 inhibitor PE 4 inhibitor Bronchodilator
Technologies	Oral formulation

Related information

COMPANY

REFERENCES

NEWS

Actions

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Reason for update on 19 March 2003

1 reference added [**482568**], one or more development status entries have been updated, one or more development status entries have been updated, indexing updated, 14 references added [**337147**, **395596**, **398255**, **401432**, **401739**, **404230**, **430164**, **448412**, **463194**, **467747**, **470041**, **470065**, **475209**, **482568**]

Summary

Altana Pharma (formerly **Byk Gulden**) was developing **pumafentrine**, a PDE-3/4 inhibitor, as a potential oral treatment for asthma and chronic obstructive pulmonary disease (COPD) [**337147**]. By February 2001, **pumafentrine** had entered phase I trials in the US for asthma and COPD [**398255**]. By March 2001, the drug was reported to be in phase II trials for asthma [**404230**]; however, in November 2002, the company reported that phase II results had not met expectations with regard to the duration of action, and that the research focus was to switch to the active metabolite, **hydroxypumafentrine** (qv), which has been shown to exhibit a longer plasma half-life than **pumafentrine** [**470041**], [**470065**]. In March 2003, Altana confirmed that research on **pumafentrine** had ceased [**482568**].

Also in November 2002, Altana stated that two backup PDE-3 inhibitors were being evaluated [**470041**], [**470065**].

Altana was also investigating **pumafentrine** for the potential treatment of bronchitis, however, no development had been reported for this indication since August 1999 [**337147**].

Development Status

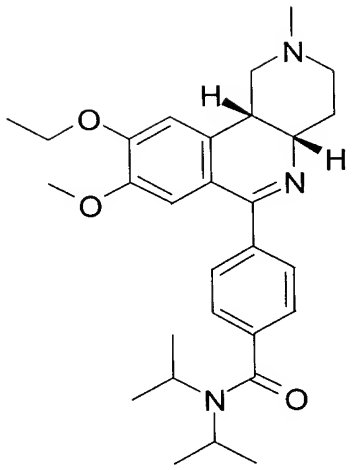
HISTORY

Detailed status for **ALTANA AG**

Indication	Country	Status	Reference	Date
Asthma	US	Discontinued	**482568**	13 November 2002
Bronchitis	Germany	Discontinued	**482568**	13 November 2002
Chronic obstructive pulmonary disease	US	Discontinued	**482568**	13 November 2002

Chemistry

Structure



Confidence Level : 1

Compound names associated with this drug

Name	Type
207993-12-2	CAS RN
pumafentrine	INN
BY-343	Research Code
pumafentrin	
BYK-33043	Research Code

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The Investigational Drugs database

DRUG report

D-4396 oder Sch 351591

Sch-351591

Company	**Celltech Group plc**
Highest Dev Status	Discontinued
Indications	Chronic obstructive pulmonary disease Inflammation Asthma
Actions	Anti-inflammatory PDE 4 inhibitor Bronchodilator

Related information

COMPANY

REFERENCES

NEWS

PATENT

Actions

EMAIL IF UPDATED

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WORD VIEW

FIND SIMILAR

Reason for update on 24 March 2004

1 reference added [**392386**], indexing updated, one or more development status entries have been updated, 2 references added [**366620**, **468711**]

Summary

Schering-Plough, under license from Celltech Group (formerly Chiroscience), was developing **Sch-351591** (D-4396), the lead compound in a series of PDE 4 inhibitors, for the potential treatment of asthma and chronic obstructive pulmonary disorder (COPD) [**323258**], [**458719**]. By January 2000, phase I studies for asthma were underway [**353474**], and by January 2002, phase I studies for COPD had been initiated [**444768**]; both trials were ongoing in July 2002 [**458718**], [**458363**], [**459631**]. However, by October 2002, Schering-Plough had discontinued development of **Sch-351591** and all rights had been returned to Celltech [**468713**], [**468711**].

By 1999, **Sch-351591** had superseded **D-4418** (qv) as previous lead in the series [**323258**], [**324364**], [**458719**].

SCH-351591

In September 2001, preclinical data were presented on **Sch-351591** at the 11th RSC-SCI Medicinal Chemistry Symposium in Cambridge, UK. In the PDE 4 and HARBS assays, **Sch-351591** displayed IC50 values of 60 and 150 nM, and inhibited TNFalpha release with an IC50 value of 68 nM. In rat, at 3 mg/kg, F was 36%, half life was 10.2 h, Cmax was 3054 ng/ml and CL was 0.3 ml/min/kg. In ascaris-sensitive primates, **Sch-351591** produced 90% inhibition of eosinophilia with no evidence of emesis, whilst in guinea-pig models, significant effects were detectable at 0.3 mg/kg. In the standard ferret models of emesis, one animal of eight wretched at 8 mg/kg, with no effect at 5 mg/kg [**422672**]. These data were published in June 2002. **Sch-351591** was found to be selective for PDE 4 over PDE 1, 2 ,3, 5 and 7 [**455450**].

By April 1999, **Sch-351591** had completed certain preclinical studies and was scheduled to enter clinical trials in 2000 [**323258**].

Sch-351591 is a related, more potent analog of **D-4418** (qv). It appears to share a similar selectivity profile against PDE 4 but lacks the emetic potential of its parent compound [**324364**].

OTHER ANALOGS

By 1998, a number of lead PDE 4 inhibitors were being evaluated [**301669**], [**303030**], [**303031**]. In a new series of quinolone-3-carboxamides identified by Chiroscience which showed IC50 values against PDE 4 of 0.46 to 49 microM, one compound was free of side effects when dosed at 20 mg/kg in the ferret emesis model [**301669**]. Another series of 3,4-dialkoxybenzenesulfonamides showed IC50 values against PDE 4 of 1.5 to 100 microM and ratios of PDE 4 inhibition to high-affinity **rolipram** binding activity down to 0.17 (**rolipram** (qv) gives a ratio of 175) [**303030**]. Also, a series of 1,3-disubstituted xanthines showed IC50 values against PDE 4 down to 0.67 microM and ratios of PDE 4 inhibition to high-affinity **rolipram** binding activity down to 4 [**303031**].

By 2000, Celltech-Chiroscience had also synthesized a series of 7-methoxybenzofuran-4-carboxamides. The 2-acetyl compound in this series was identified as a lead due to its good oral activity in a functional model of inflammation and at doses which showed none of the CNS and emetic side effects of **rolipram** [**382308**].

By 2002, a series of 7-methoxyfuro[2,3-c]pyridines were synthesized and evaluated by Celltech for good selectivity for the catalytic site over **rolipram** binding site. 2-Ethyl-7-methoxyfuro[2,3-c]pyridine-4-carboxylic acid (3,5-dichloro-1-oxopyridin-4-yl)amide was identified as the most potent compound with an IC50 value of 0.014 microM. In vivo, the compound had a good pharmacokinetic profile showing a Cmax of 1411 ng/ml and AUC 4942 ng h/ml in guinea pigs when dosed at 3 mg/kg po. The bioavailability of the

compound was found to be 54%. It also displayed 40% inhibition of lung eosinophilia when administered po at 10 mg/kg [**444451**].

ADDITIONAL INFORMATION

Chiroscience and Schering-Plough were collaborating on the development of PDE 4 inhibitors for the potential treatment of asthma and other inflammatory disorders since 1997 [**251730**]. By October 2002, the agreement had been terminated [**468711**]. Celltech has also, in collaboration with Merck, developed other lead PDE 4 inhibitors, distinct from those under co-development with Schering-Plough (PDE 4 inhibitors, Celltech/Merck; qv) [**366620**].

In December 1999, Lehman Brothers predicted that **Sch-351591** had a 20% chance of reaching the market, with a possible launch in 2005 and potential peak sales of $500 million in 2010 [**352078**]. In November 2000, Lehman Brothers predicted a 2005 launch, with estimated peak sales of $500 million in 2010 and a 20% probability of reaching the market [**352078**], [**394921**].

Development Status

HISTORY

Detailed status for **Celltech Group plc**

Indication	Country	Status	Reference	Date
Asthma	US	Discontinued	**468717**	01 November 2002
Chronic obstructive pulmonary disease	US	Discontinued	**468717**	01 November 2002

Detailed status for **Schering-Plough Corp**

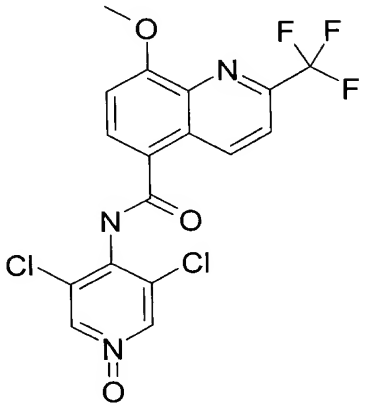
Indication	Country	Status	Reference	Date
Asthma	US	Discontinued	**468713**	01 November 2002
Asthma	Western Europe	Discontinued	**468713**	01 November 2002
Chronic obstructive pulmonary disease	US	Discontinued	**468713**	01 November 2002
Chronic obstructive pulmonary disease	Western Europe	Discontinued	**468713**	01 November 2002

Licensing

Summary	Reference
Schering-Plough Corp	
A joint development agreement for PDE 4 inhibitors between Schering-Plough and Chiroscience (now Celltech) was announced in June 1997. Schering-Plough was to acquire exclusive worldwide rights to all products derived from the collaboration and was to have access to all Chiroscience PDE 4 inhibitors, including **D-4418** (qv). Chiroscience was to research new PDE 4 inhibitors and conduct medicinal chemistry and in vitro biological screening. Schering-Plough was to conduct secondary in vitro and in vivo studies, and would then be responsible for all product development, manufacturing and marketing [**251730**]. In April 1999, Schering-Plough made a milestone payment to Chiroscience relating to **Sch-351591** [**323258**]. By October 2002, the agreement had been terminated with all rights reverting to Celltech [**468711**].	**251730**

Chemistry

Structure



Confidence Level : 1

Compound names associated with this drug

Name	Type
D-4396	Research Code
PDE 4 inhibitors, Schering-Plough/Chiroscience	
Sch-351591	Research Code
PDE 4 inhibitors, Schering-Plough/Celltech	

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Metabolism of a novel phosphodiesterase-IV inhibitor (V11294) by human hepatic cytochrome P450 forms

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Abstract:

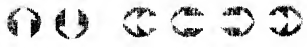
1. The metabolism of a novel phosphodiesterase-IV inhibitor (V11294) was studied in human liver microsomal and cytosol preparations and in cDNA-expressed human hepatic CYP forms.
2. Human liver microsomes, but not cytosol, catalysed the NADPH-dependent metabolism of V11294 to V10331 (formed by hydroxylation of the cyclopentyl ring), V10332 (*N*-desethyl V11294) and V11689 (formed by hydroxylation of the isopropyl side chain). In addition, smaller amounts of a secondary metabolite V11690 (which can be formed from either V10332 or V11689) were also produced.
3. Kinetic analysis of V11294 metabolism to V10331, V10332 and V11689 in two preparations of pooled human liver microsomes revealed average K_m = 2.5, 8.1 and 3.9 μM, respectively.
4. The metabolism of V11294 was determined with a characterized bank of 16 individual human liver microsomal preparations employing a V11294 substrate concentration of 8μM (i.e. approximately the K_m for V10332

formation and around twice the K_m for V10331 and V11689 formation). Good correlations (r^2 = 0.570-0.903) were observed between V10331, V10332 and V11689 formation and markers of CYP3A forms. In contrast, poorer correlations (r^2 = 0.0002-0.428) were observed with markers of CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP4A9/11.

5. Using human B-lymphoblastoid cell microsomes containing cDNA-expressed CYP forms, V11294 (8 µM) was metabolized by cDNA-expressed CYP3A4 to V10331, V10332 and V11689, with lower amounts of V11690 also being formed. Lower rates of V11294 metabolism to some V11294 metabolites were also observed with cDNA-expressed CYP2C9, CYP2C19 and CYP2D6, whereas only very low or undetectable rates of V11294 metabolism were observed with cDNA-expressed CYP1A2, CYP2A6, CYP2B6, CYP2C8 and CYP2E1.

6. The metabolism of V11294 (8 µM) to V10331, V10332 and V11689 was markedly inhibited by the CYP3A mechanism-based inhibitor troleandomycin. In contrast, V11294 metabolism was not significantly affected by inhibitors of CYP1A2, CYP2C9, CYP2D6 and CYP2E1 or by the CYP2C19 substrate *S*-mephenytoin.

7. In summary, by correlation analysis, chemical inhibition studies and the use of cDNA-expressed CYPs, V11294 metabolism in human liver to V10331, V10332 and V11689 appears to be primarily catalysed by CYP3A forms.



XENOBIOTICA, 2002, VOL. 32, NO. 6, 521–534

Taylor&Francis
healthsciences

Metabolism of a novel phosphodiesterase-IV inhibitor (V11294) by human hepatic cytochrome P450 forms

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Received 17 September 2001

1. The metabolism of a novel phosphodiesterase-IV inhibitor (V11294) was studied in human liver microsomal and cytosol preparations and in cDNA-expressed human hepatic CYP forms.

2. Human liver microsomes, but not cytosol, catalysed the NADPH-dependent metabolism of V11294 to V10331 (formed by hydroxylation of the cyclopentyl ring), V10332 (*N*-desethyl V11294) and V11689 (formed by hydroxylation of the isopropyl side chain). In addition, smaller amounts of a secondary metabolite V11690 (which can be formed from either V10332 or V11689) were also produced.

3. Kinetic analysis of V11294 metabolism to V10331, V10332 and V11689 in two preparations of pooled human liver microsomes revealed average K_m = 2.5, 8.1 and 3.9 μM, respectively.

4. The metabolism of V11294 was determined with a characterized bank of 16 individual human liver microsomal preparations employing a V11294 substrate concentration of 8 μM (i.e. approximately the K_m for V10332 formation and around twice the K_m for V10331 and V11689 formation). Good correlations (r^2 = 0.570–0.903) were observed between V10331, V10332 and V11689 formation and markers of CYP3A forms. In contrast, poorer correlations (r^2 = 0.0002–0.428) were observed with markers of CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP4A9/11.

5. Using human B-lymphoblastoid cell microsomes containing cDNA-expressed CYP forms, V11294 (8 μM) was metabolized by cDNA-expressed CYP3A4 to V10331, V10332 and V11689, with lower amounts of V11690 also being formed. Lower rates of V11294 metabolism to some V11294 metabolites were also observed with cDNA-expressed CYP2C9, CYP2C19 and CYP2D6, whereas only very low or undetectable rates of V11294 metabolism were observed with cDNA-expressed CYP1A2, CYP2A6, CYP2B6, CYP2C8 and CYP2E1.

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7. In summary, by correlation analysis, chemical inhibition studies and the use of cDNA-expressed CYPs, V11294 metabolism in human liver to V10331, V10332 and V11689 appears to be primarily catalysed by CYP3A forms.

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Introduction

Members of the cytochrome P450 (CYP) superfamily are known to play a major role in the oxidative metabolism of both xenobiotics and certain endogenous compounds (Parkinson 1996). Human hepatic CYP1A, CYP2A, CYP2B, CYP2C, CYP2D, CYP2E and CYP3A forms can metabolize a wide variety of therapeutic agents (Wrighton *et al.* 1993, Nelson *et al.* 1996, Parkinson 1996, Pelkonen *et al.* 1998).

V11294 (3-(3-cyclopentyloxy-4-methoxybenzyl)-6-ethylamino-8-isopropyl-3*H*-purine) is a novel phosphodiesterase-IV inhibitor which was under development as an anti-asthma agent. The inhibition of phosphodiesterase-IV by such agents leads to increased levels of cAMP in the affected cells (smooth muscle, macrophages and other immune cells), which initiates a cascade of physiological responses including bronchorelaxation and reduction in the immune response to different stimuli (Giembycz 2000).

V11294 has been administered to human subjects at oral dose levels of 25–300 mg and has been shown to be metabolized to various products (Subrahmanyam and Tonelli, unpublished observations), including *N*-desethyl V11294 (metabolite V10332). The structure of V11294 and some pathways of V11294 metabolism are shown in figure 1.

The objective of the current study was to elucidate the CYP forms responsible for V11294 metabolism in human liver. 'Reaction phenotyping' studies (Parkinson 1996) were performed by correlation analysis, chemical inhibition and the use of cDNA-expressed human CYP forms.

Materials and methods

Materials

NADPH, Tris, diethyldithiocarbamate, quinidine, sulfaphenazole and troleandomycin were obtained from Sigma-Aldrich Co. (Poole, UK) and furafylline and *S*(+)-mephenytoin from Salford Ultrafine Chemicals and Research (Manchester, UK). Samples of V11294, V10331, V10332, V11689, V11690 and V10388 (internal standard for high-performance liquid chromatography [HPLC]) were supplied by Department of Drug Metabolism and Pharmacokinetics, Purdue Pharma L.P. (Ardsley, NY, USA). A reaction phenotyping kit (Product No. H0500), containing 16 separate human liver microsomal preparations characterized for total CYP content and a range of CYP form enzyme activities (table 2), was purchased from XenoTech LLC (Kansas City, KS, USA) and stored at −80°C. Samples of microsomes from human B-lymphoblastoid cell lines engineered to express stably cDNAs for human cytochrome P450 forms (GENTEST Corporation, Woburn, MA, USA) were obtained from Cambridge Bioscience (Cambridge, UK). The samples of GENTEST cell microsomes comprised control cell microsomes (i.e. no transfected human cytochrome P450 form cDNA but contains native CYP1A1 activity) and cell microsomes containing CYP1A2, CYP2A6 + OR (i.e. CYP2A6 cDNA plus human NADPH-cytochrome P450 reductase (OR) cDNA), CYP2B6, CYP2C8 + OR, CYP2C9 + OR, CYP2C19, CYP2D6 + OR, CYP2E1 + OR and CYP3A4 + OR.

Human liver microsomes

Samples of human liver were transported on ice to TNO BIBRA and stored at −80°C. The Research Ethics Committee of King's College Hospital (London, UK) granted approval for the use of this tissue, which was surplus to clinical requirements. Washed liver microsomal fractions were prepared in 0.154 M KCl containing 50 mM Tris-HCl as described (Lake 1987). Washed microsomal fractions were assayed for total CYP content (Lake 1987) and for protein (Lowry *et al.* 1951) employing bovine serum albumin as standard. The washed microsomal fractions were diluted to 10 mg ml^{-1} and aliquots stored at −80°C. Two separate batches, designated pools A and B, of pooled human liver microsomes were prepared, with each batch consisting of pooled liver samples from five subjects. Pool A comprised liver samples from males aged 15, 17 and 29 years and females aged 31 and 42 years, whereas pool B comprised liver samples, from males aged 2.5 and 58 years and females, aged 14, 59 and 74 years.

Metabolism of V11294 by human liver microsomes

The NADPH-dependent metabolism of V11294 was studied in incubation mixtures containing 0–100 μM V11294 (added in 5 μl methanol), 10 mM $MgCl_2$, 2 mM EDTA, 80 mM phosphate buffer pH 7.4 and 0.5–2.0 mg microsomal protein in a final volume of 1 ml. After 5-min pre-incubation at 37 °C in a shaking water bath, the reaction was initiated by the addition of 1 mM NADPH. Incubations were performed for 5–30 min at 37 °C and were terminated by the addition of 3 ml ice-cold acetonitrile, followed by the addition of 0.05 ml 0.8 mM V10388 (internal standard) in methanol. Blank incubations contained all components except NADPH, which was added after the reaction was terminated with acetonitrile. For each V11294 substrate concentration, incubation time and microsomal protein concentration, incubations were conducted in duplicate with a single blank (no NADPH) tube. Tubes were left on ice for 30 min and then centrifuged at 1730*g* average for 10 min at 4 °C. A 3-ml sample of the supernatant was removed into a clean glass tube and the solvent removed in a gyrovap at 50 °C for 60–90 min. The residue in each tube was reconstituted with either 1 or 2 ml HPLC mobile phase (see below), centrifuged at 10 000*g* for 5 min in a benchtop centrifuge and subsequently filtered through a 10 mm 0.2 μm Anotop 10 Plus syringe filter (Whatman International, Maidstone, UK). The samples were then stored at 4 °C before HPLC analysis. The HPLC method employed was based on a fully validated procedure for analysis of V11294 and V11294 metabolites in human plasma developed by Purdue Pharma L.P. (Ardsley, NY, USA). Chromatography of 50 μl aliquots of the filtered incubate extracts was performed with a 150 × 2.1 mm Waters Symmetry C8 3.5 μm column (Catalogue No. WAT 106011) protected by a 10 × 2.1 Waters Symmetry C8 3.5 μm guard column (WAT 106128) and isocratic elution with a mobile phase of 27% (v/v) acetonitrile in water containing 0.2% (v/v) glacial acetic acid and 0.1% triethylamine. Elution was achieved at a flow rate of 0.4 ml min^{-1} with a column temperature of 40 °C. The eluent was monitored at 290 nm. Retention times of V10331, V11690, V10332, V11689, V10388 (internal standard) and V11294 were approximately 3.5, 6.2, 11.7, 13.3, 22.8 and 25.9 min, respectively.

Metabolism of V11294 by human liver cytosol

Incubation mixtures contained 1 mM NADPH, 10 mM $MgCl_2$, 2 mM EDTA, 1 mg protein and 80 mM phosphate buffer pH 7.4 in a final volume of 1 ml. After a 5-min pre-incubation at 37 °C in a shaking water bath, the reaction was initiated by adding 100 μM V11294 (added in 5 μl methanol). Incubations were also performed in the absence of NADPH. Duplicate incubations were terminated after 15, 30 and 60 min with 3 ml ice-cold acetonitrile, followed by the addition of 0.05 ml 0.4 mM V10388 (internal standard) in methanol. For these studies, blank incubations comprised tubes stopped at zero time. Incubations were processed for HPLC analysis as described above.

V11294 metabolism correlation analysis

Incubation mixtures contained 8 μM V11294 (added in 5 μl methanol), 10 mM $MgCl_2$, 2 mM EDTA, 0.5 mg microsomal protein and 87 mM phosphate buffer pH 7.4 in a final volume of 1 ml. After 5-min pre-incubation at 37 °C in a shaking water bath, the reaction was initiated by addition of 1 mm (final concentration) NADPH. Incubations were conducted in triplicate with a single blank (no NADPH) for each of the 16 preparations of characterized human liver microsomes and were terminated after 5 min by addition of 3 ml ice-cold acetonitrile, followed by the addition of 0.05 ml 0.8 mM V10388 (internal standard) in methanol. Incubations were processed for HPLC analysis as described above.

V11294 metabolism by cDNA-expressed CYP forms

Incubation mixtures contained 8 μM V11294 (added in 0.25 ml 100 mM Tris-HCl buffer pH 7.4) 10 mM $MgCl_2$, 2 mM EDTA, 1 mM NADPH and 80 mM Tris-HCl buffer pH 7.4 in a final volume of 0.5 ml. After 5-min pre-incubation in a 37 °C water bath, the reaction was initiated by adding 0.5 mg GENTEST cell microsomal protein with gentle mixing. The incubations were conducted in duplicate with a single blank (no NADPH) for each CYP form preparation. Reactions were terminated after 20, 45 and 120 min with 1.5 ml ice-cold acetonitrile, followed by 0.05 ml 0.8 mM V10388 (internal standard) in methanol. Incubations were processed for HPLC analysis as described above.

V11294 metabolism inhibition studies

For the compounds requiring pre-incubation with NADPH, incubation mixtures contained 10 mM $MgCl_2$, 2 mM EDTA, 1 mM NADPH, 85 mM phosphate buffer pH 7.4, 0.5 mg microsomal protein and either furafylline (5–50 μM), diethyldithiocarbamate (5–100 μM) or troleandomycin (5–100 μM) in a volume of 0.85 ml. All the inhibitors were added in DMSO (5 μl/tube) and the concentrations refer to the final concentrations in a 1 ml incubation. Following a 30 min pre-incubation at 37 °C in a shaking water bath, 8 μM V11294 (added in 0.1 ml 100 mM phosphate buffer pH 7.4) and another aliquot of NADPH (final concentration 2 mM) were added. Incubations were performed for 5 min at 37 °C and were terminated by the addition of 3 ml ice-cold acetonitrile, followed by 0.05 ml 0.8 mM V10388

(internal standard) in methanol. All test incubations were performed in either triplicate (no inhibitor added but containing 5 µl DMSO/tube) or duplicate (for each concentration of each inhibitor), with single blank tubes where V11294 was added after the ice-cold acetonitrile. Incubations were processed for HPLC analysis as described above. For the compounds not requiring pre-incubation with NADPH, incubation mixtures contained 8 µM V11294 (added in 0.1 ml 100 mM phosphate buffer pH 7.4), 10 mM $MgCl_2$, 2 mM EDTA, 0.5 mg microsomal protein, 85 mM phosphate buffer pH 7.4 and either sulfaphenazole (2–50 µM), quinidine (2–20 µM) or *S*-mephenytoin (50–500 µM) in a volume of 0.95 ml. All the inhibitors were added in DMSO (5 µl/tube) and the concentrations refer to the final concentrations in a 1 ml incubation. Following a 10 min pre-incubation at 37 °C in a shaking water bath, 1 mM NADPH was added. Incubations were performed for 5 min at 37 °C and were terminated by the addition of 3 ml ice-cold acetonitrile, followed by 0.05 ml 0.8 mM V10388 (internal standard) in methanol. All test incubations were performed in either triplicate (no inhibitor added but containing 5 µl DMSO/tube) or duplicate (for each concentration of each inhibitor), with single blank tubes where NADPH was added after the ice-cold acetonitrile. Incubations were processed for HPLC analysis as described above.

Results

Kinetics of V11294 metabolism

Initial V11294 metabolism studies were conducted with 100 µM substrate and the pool A and B human liver microsomal preparations. V11294 was metabolized by human liver microsomes in the presence of NADPH to a number of metabolites. The major metabolites detected were V10331 (formed by hydroxylation of the cyclopentyl ring), V10332 (*N*-desethyl V11294) and V11689 (formed by hydroxylation of the isopropyl side chain), together with smaller amounts of the secondary metabolite V11690, which can be formed from either V10332 or V11689 (figure 1). With both human liver microsomal preparations, V11294 metabolism to V10331, V10332 and V11689 was linear with respect to incubation time for up to 10 min and microsomal protein concentration up to 1.5 mg protein ml^{-1} (data not shown).

V11294 was incubated at a substrate concentration of 100 µM in both the presence and absence of 1 mm NADPH with 1 mg of cytosolic protein from both the A and B human liver pools for up to 60 min. Under these conditions only trace amounts ($\leqslant$ 1 pmol per incubation) of V10331, V10332, V11689 and V11690 were formed (data not shown). These data suggest that cytosolic enzymes do not contribute to V11294 metabolism.

Using the pool A and B human liver microsomal preparations, the metabolism of V11294 was investigated over a substrate concentration range of 0.5–50 µM. Kinetic analysis of Eadie–Hofstee plots revealed K_m for V10331, V10332 and V11689 formation between 1.8 and 11.9 µM (table 1). The pool B human liver microsomal preparation was more active than the pool A preparation as indicated by the higher V_{max} (expressed per unit of microsomal protein) for formation of V10331, V10332 and V11689. The total CYP contents of the pool A and B human liver microsomal preparations were 0.39 and 0.72 nmol mg^{-1} protein, respectively. When V_{max} was expressed per unit of total CYP, the differences between the two pools were much less marked (table 1).

Average K_m was for V11294 metabolism to V10331, V10332 and V11689 by the pool A and B human liver microsomal preparations were 2.5, 8.1 and 3.9 µM, respectively (table 1). For subsequent studies, a V11294 substrate concentration of 8 µM was selected as being approximately the K_m for V10332 formation and around twice K_m for V10331 and V11689 formation. This substrate concentration

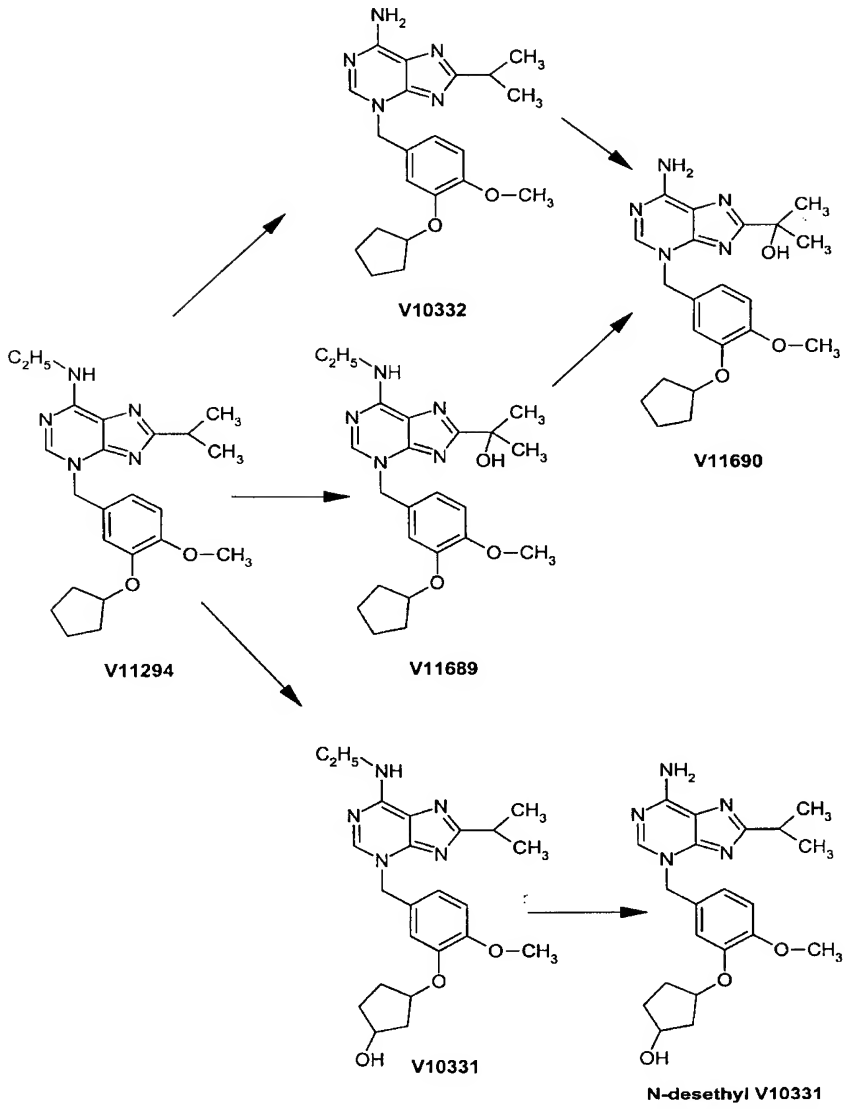

Figure 1. Some pathways of V11294 metabolism.

was also similar to plasma levels of V11294 observed after oral administration to human subjects (unpublished observations).

V11294 metabolism correlation analysis

The metabolism of V11294 was examined with a characterized panel of 16 human liver microsomal preparations employing a V11294 substrate concentration of 8 μM. V11294 was metabolized to V10331, V10332 and V11689 by all 16 human liver microsomal preparations examined (figure 2). Some of these microsomal preparations were more active than the pool A and B human liver microsomal

Table 1. Kinetic analysis of V11294 metabolism to V10331, V10332 and V11689 in two preparations of human liver microsomes.

Microsomal preparation	V11294 metabolism kinetics[a]								
	K_m (μM)			V_{max} (pmol min^{-1} mg^{-1} protein)			V_{max} (pmol min^{-1} $nmol^{-1}$ CYP)		
	V10331	V10332	V11689	V10331	V10332	V11689	V10331	V10332	V11689
Pool A	3.1	4.3	3.0	68.0	68.3	47.0	174	175	121
Pool B	1.8	11.9	4.7	98.5	163.1	108.4	137	227	151
Average	2.5	8.1	3.9	83.3	115.7	77.7	156	201	136

[a] Duplicate incubations were performed with each V11294 substrate concentration and 1 mg microsomal protein for 10 min.

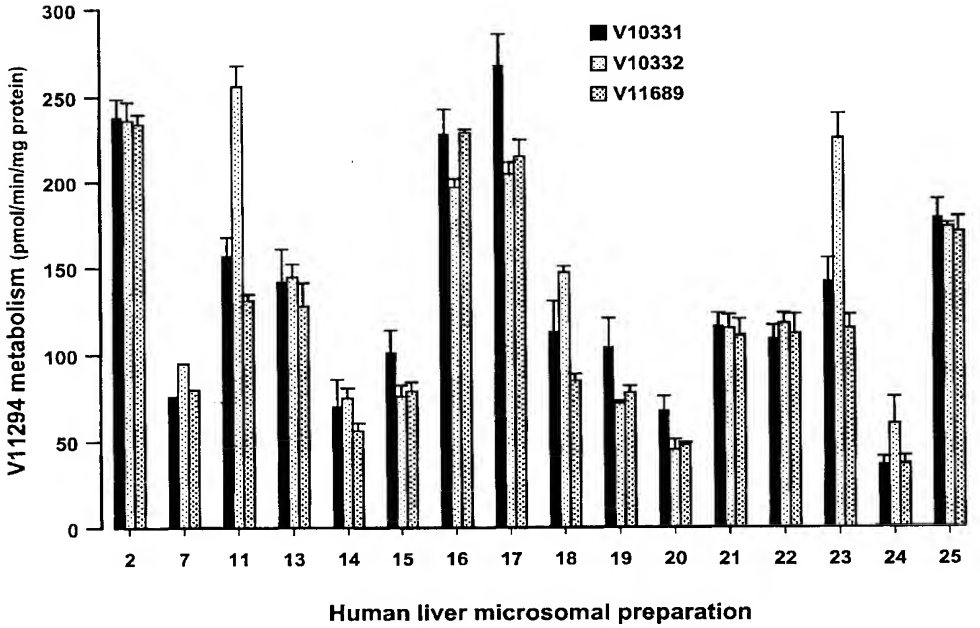

Figure 2. Variability in the metabolism of 8 μM V11294 to V10331, V10332 and V11689 in a panel of 16 individual human liver microsomal preparations. Incubations were performed with 0.5 mg protein for 5 min. Results are either the average of duplicate incubations (preparation number 7) or as mean ± SEM of triplicate incubations (all other preparations).

preparations. With the majority of the 16 characterized human liver microsomal preparations examined, similar amounts of V10331, V10332 and V11689 were formed. Although preparations 11 and 23 produced somewhat greater quantities of V10322 (figure 2), this could not be attributed to any obvious differences in the CYP enzyme profiles of these two preparations compared to those of the other liver microsomal preparations examined (data not shown). In 12 of the 16 microsomal preparations examined, smaller amounts of the secondary metabolite V11690 ranging from 1 to 57 pmol min^{-1} mg^{-1} protein were also formed (data not shown).

The rates of V11294 metabolism to V10331, V10332 and V11689 were correlated with data for total CYP content and a range of CYP form-dependent enzyme activities in the 16 human liver microsomal preparations. The best correlations (table 2) for V11294 metabolism to V10331, V10332 and V11689 were observed with total CYP content (r^2 = 0.559–0.654) and two markers of CYP3A forms, namely testosterone 6β-hydroxylase (r^2 = 0.776–0.903) and dextromethorphan *N*-demethylase (r^2 = 0.570–0.710). For V11294 metabolism to V10331, V10332 and V11689 and CYP form markers for CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP4A9/11, r^2 of 0.0002–0.428 were observed (table 2). These latter enzyme activities comprised 7-ethoxyresorufin *O*-deethylase, coumarin 7-hydroxylase, *S*-mephenytoin *N*-demethylase, taxol 6α-hydroxylase, tolbutamide methylhydroxylase, *S*-mephenytoin 4′-hydroxylase, dextromethorphan *O*-demethylase, chlorzoxazone 6-hydroxylase and 4-nitrophenol hydroxylase and lauric acid 12-hydroxylase, respectively.

Table 2. Correlation analysis for V11294 metabolism to V10331, V10332 and V11689 with various CYP-dependent enzyme activities in human liver microsomes.

CYP form	Enzyme activity[a]	V11294 metabolism correlation (r^2)		
		V10331	V10332	V11689
–	total CYP content	0.652	0.654	0.559
1A2	7-ethoxyresorufin *O*-deethylase	0.001	0.072	0.0002
2A6	coumarin 7-hydroxylase	0.345	0.183	0.277
2B6	*S*-mephenytoin *N*-demethylase	0.380	0.286	0.428
2C8	taxol 6α-hydroxylase	0.329	0.424	0.309
2C9	tolbutamide methylhydroxylase	0.290	0.055	0.271
2C19	*S*-mephenytoin 4′-hydroxylase	0.137	0.049	0.121
2D6	dextromethorphan *O*-demethylase	0.042	0.061	0.035
2E1	chlorzoxazone 6-hydroxylase	0.102	0.036	0.115
	4-nitrophenol hydroxlyase	0.304	0.103	0.295
3A4/5	testosterone 6β-hydroxylase	0.873	0.776	0.903
	dextromethorphan *N*-demethylase	0.694	0.570	0.710
4A9/11	lauric acid 12-hydroxylase	0.020	0.019	0.016

[a] Total CYP content and the enzyme activities shown were correlated with data obtained from the metabolism of 8 μM V11294 to V10331, V10332 and V11689 (figure 2) in a panel of 16 individual human liver microsomal preparations.

Metabolism of V11294 by cDNA-expressed CYP forms

The metabolism of 8 μM V11294 was examined in GENTEST B-lymphoblastoid cell microsomes containing cDNA-expressed CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4, together with microsomes from control (wild type) cells which contain native CYP1A1. V11294 was metabolized in a time-dependent manner by B-lymphoblastoid cell microsomes containing cDNA-expressed CYP2C9, CYP2C19, CYP2D6 and CYP3A4 (data not shown). With the cDNA-expressed CYP3A4 preparation V11294 was metabolized to V10331, V10332, V11689 and V11690, the former three V11294 metabolites being produced in much greater amounts than V11690. V11294 was also metabolized to V10331, V10332 and V11689 by the cDNA-expressed CYP2C9 preparation, with a small quantity of V11690 also being formed after 120 min of incubation. The cDNA-expressed CYP2D6 preparation metabolized V11294 to V10331 and V10332, with a small amount of V11689 being formed after 120 min of incubation. With the cDNA-expressed CYP2C19 preparation, V10332 was the primary metabolite of V11294, with a trace amount of V10331 being formed after 120 min of incubation. Data for 20-min incubations with the cDNA-expressed CYP2C9, CYP2C19, CYP2D6 and CYP3A4 preparation is shown in figure 3. Overall, the CYP3A4 preparation was the most active CYP form examined.

In contrast to the above cDNA-expressed CYP forms, V11294 was not metabolized by control B-lymphoblastoid cell microsomes or by microsomes containing cDNA-expressed CYP1A2, CYP2B6, CYP2C8 and CYP2E1 (data not shown). With cDNA-expressed CYP2A6, trace amounts of V10332 (< 0.5 pmol min^{-1} mg^{-1} protein) were formed after 120 min of incubation.

Inhibition of V11294 metabolism

The effect of some known human CYP inhibitors and one substrate (*S*-mephenytoin) on the metabolism of 8 μM V11294 in human liver microsomes was

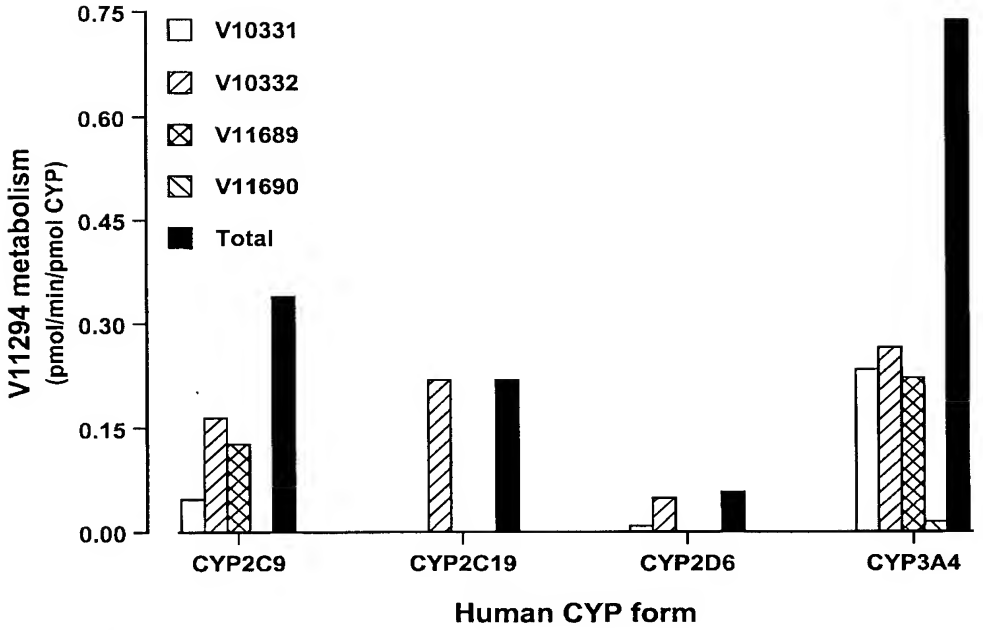

Figure 3. Metabolism of 8 μM V11294 by cDNA-expressed CYP2C9, CYP2C19, CYP2D6 and CYP3A4. Results are the averages of duplicate 20-min incubations with 0.5 mg B-lymphoblastoid cell microsomal protein expressed as pmol V11294 metabolite min^{-1} $pmol^{-1}$ CYP. Total metabolism refers to the sum of V11294 metabolism to V10331, V10332, V11689 and V11690.

studied. For the mechanism-based inhibitors (Newton *et al.* 1995) furafylline (CYP1A2), diethyldithiocarbamate (CYP2E1) and troleandomycin (CYP3A4), the compounds were pre-incubated with NADPH and the liver microsomes for 30 min at 37 °C prior to the addition of 8 μM V11294 and another aliquot of NADPH. In the studies with the inhibitors sulfaphenazole (CYP2C9) and quinidine (CYP2D6) and the substrate *S*-mephenytoin (CYP2C19), the compounds were pre-incubated with liver microsomes for 10 min at 37 °C prior to the addition of 8 μM V11294 and NADPH. The pool B human liver microsomal preparation was used for these studies, except for the investigations with troleandomycin where both the pool A and B microsomal preparations were employed. None of the CYP inhibitors and the one CYP substrate appeared to interfere with the HPLC analysis of V11294 metabolism to V10332 and V11689. However, an interfering component present in incubations containing sulfaphenazole precluded the determination of the effect of this inhibitor on V11294 metabolism to V10331.

Treatment with all five CYP inhibitors and the one CYP substrate studied had similar effects on the three pathways of V11294 metabolism examined. V11294 metabolism to V10332 was not markedly affected by furafylline and diethyldithiocarbamate (figure 4A) and by sulfaphenazole, *S*-mephenytoin and quinidine (figure 4B). Similarly, furafylline, diethyldithiocarbamate, *S*-mephenytoin and quinidine did not markedly inhibit V11294 metabolism to V10331 and V11689 and sulfaphenazole did not markedly inhibit V11294 metabolism to

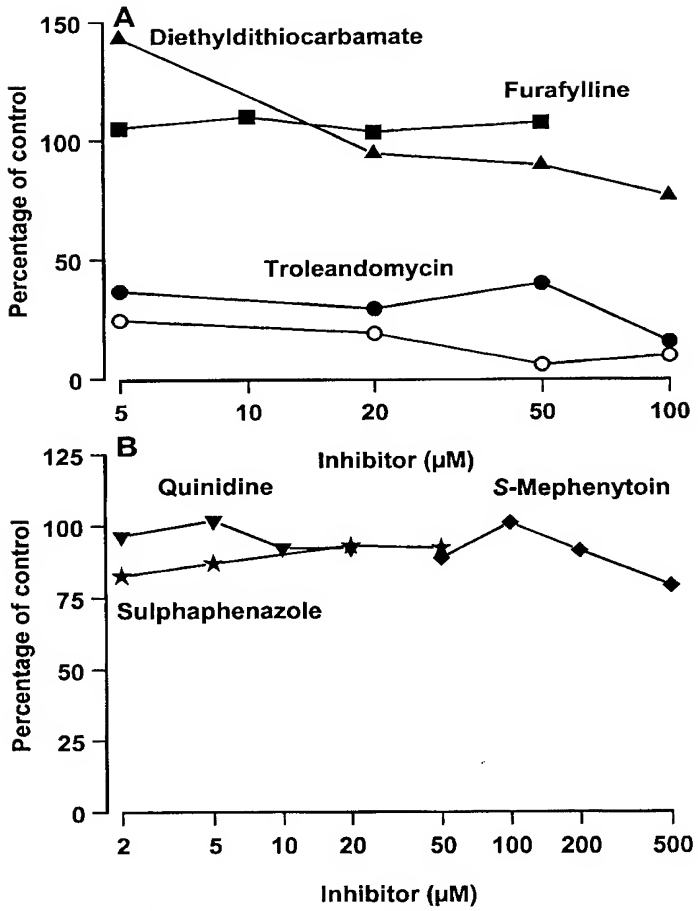

Figure 4. Inhibition of 8 μM V11294 metabolism to V10332 in human liver microsomes. To study the effects of 5–50 μM furafylline (■), 5–100 μM diethyldithiocarbamate (▲) and 5–100 μM troleandomycin (○●) (A), but not 2–50 μM sulfaphenazole (⋆), 50–500 μM *S*-mephenytoin (◆) and 2–20 μM quinidine (▼) (B), the compounds were pre-incubated with liver microsomes and NADPH for 30 min at 37°C prior to the addition of 8 μM V11294. Duplicate incubations were performed with 0.5 mg of either the pool A (open symbols) or B (closed symbols) microsomal preparations for 5 min, and the results expressed as the percentage of triplicate control (no inhibitor added) incubations. Control (no inhibitor added) rates of V11294 metabolism to V10332 were 53 and 123 pmol min^{-1} mg^{-1} protein for the pool A and B microsomal preparations, respectively.

V11689 (data not shown). In contrast, 5 μM troleandomycin markedly inhibited V11294 metabolism to V10332 to 25 and 37% of control in the pool A and B microsomal preparations, respectively (figure 4A). Treatment with 20–100 μM troleandomycin inhibited V11294 metabolism to V10332 to 5–19 and 15–40% of control in the pool A and B microsomal preparations, respectively (figure 4A). V11294 metabolism to both V10331 and V11689 was inhibited by 20–100 μM troleandomycin to 0–17 and 2–24% of control in the pool A and B microsomal preparations, respectively (data not shown).

Discussion

In this study the metabolism of V11294 by human hepatic microsomal and cytosolic fractions and cDNA-expressed human hepatic CYP isoforms has been investigated. The NADPH-dependent metabolism of V11294 to the major metabolites V10331, V10332 and V11689 was observed in two preparations of pooled human liver microsomes and in 16 individual preparations. In addition, V11690 was also identified as a minor secondary metabolite of V11294 in human liver microsomes. V11294 can thus be metabolized by human liver microsomes by *N*-deethylation, hydroxylation of the isopropyl side chain and hydroxylation of the cyclopentyl ring to yield V10332, V11689 and V10331, respectively (figure 1). Both V10332 and V11689 can be further oxidized to yield the minor secondary metabolite V11690 (figure 1). Kinetic analysis of microsomal V11294 metabolism revealed similar K_m for V10331, V10332 and V11689 formation, with a mean of 4.8 μM (table 1). Whereas V11294 was metabolized by human liver microsomal fractions to various products, the compound was not metabolized to any significant extent by cytosolic fractions in either the presence or absence of NADPH.

To identify the CYP forms responsible for V11294 metabolism in human liver, 'reaction phenotyping' studies were performed (Parkinson 1996, Clarke 1998). Such investigations can include the use of correlation analysis, chemical inhibitors, inhibitory antibodies and cDNA-expressed CYP forms. For the current study, 'reaction phenotyping' was performed by correlation analysis with a panel of characterized microsomal preparations, chemical inhibition and the use of cDNA-expressed human CYP forms. A V11294 substrate concentration of 8 μM was selected as being approximately the K_m for V10332 formation and around twice K_m for V10331 and V11689 formation. In addition, this V11294 substrate concentration is similar to the plasma C_{max} of 2.4 μM observed after oral administration of a 200 mg dose of V11294 to human subjects (Subrahmanyam and Tonelli, unpublished observations).

The metabolism of 8 μM V11294 was determined in a panel of 16 characterized human liver microsomal preparations. Good correlations were obtained between V11294 metabolism to V10331, V10332 and V11689 and testosterone 6β-hydroxylase ($r^2 = 0.776$–0.903) and dextromethorphan *N*-demethylase ($r^2 = 0.570$–0.710) activities. Both these enzyme activities are considered to be relatively specific markers for CYP3A forms in human liver (Wrighton *et al.* 1993, Gorski *et al.* 1994, Parkinson 1996, Clarke 1998, Pelkonen *et al.* 1998). V11294 biotransformation to all three primary metabolites also correlated with total CYP content ($r^2 = 0.559$–0.654). This may be attributable to the fact that CYP3A forms account for around 20–50% of the total CYP content of human liver (Wrighton *et al.* 1993, Shimada *et al.* 1994, Rodrigues 1999). In addition, while V_{max} (expressed per unit protein) for V11294 metabolite formation was higher in the pool B human liver microsomal preparation, when expressed per unit of total CYP V_{max} was similar in the pool A and B microsomal preparations. In contrast, to the markers of CYP3A forms and total CYP content, poorer correlations ($r^2 = 0.0002$–0.428) were observed between V11294 metabolism and 7-ethoxyresorufin *O*-deethylase, coumarin 7-hydroxylase *S*-mephenytoin *N*-demethylase, taxol 6α-hydroxylase, tolbutamide methylhydroxylase, *S*-mephenytoin 4′-hydroxylase, dextromethorphan *O*-demethylase, chlorzoxazone 6-hydroxylase and 4-nitrophenol hydroxylase and lauric acid 12-hydroxylase. These latter enzyme activities are considered markers of CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19,

CYP2D6, CYP2E1 and CYP4A9/11, respectively (Wrighton *et al.* 1993, Heyn *et al.* 1996, Parkinson 1996, Clarke 1998, Pelkonen *et al.* 1998).

The conclusions of the correlation analysis study are supported by the results of the chemical inhibition studies. Previous work has demonstrated that furafylline, sulfaphenazole, *S*-mephenytoin, quinidine, diethyldithiocarbamate and troleandomycin may be considered either inhibitors or substrates of human hepatic CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4, respectively (Wrighton *et al.* 1993, Chang *et al.* 1994, Clarke *et al.* 1994, Baldwin *et al.* 1995, Newton *et al.* 1995, Ono *et al.* 1996, Parkinson 1996, Clarke 1998, Pelkonen *et al.* 1998, Tucker *et al.* 2001). The substrate *S*-mephenytoin was employed as a CYP2C19 inhibitor owing to the lack of specific chemical inhibitor for this CYP form (Clarke 1998, Tucker *et al.* 2001). Using two separate human liver microsomal preparations (pools A and B), 20–100 μM troleandomycin inhibited V11294 metabolism to V10331, V10332 and V11689 to 0–40% of control. Previous studies have demonstrated that troleandomycin is a relatively specific mechanism-based inhibitor of CYP3A forms in human liver (Chang *et al.* 1994, Newton *et al.* 1995, Ono *et al.* 1996). Unlike the effect of troleandomycin, 5–50 μM furafylline, 5–100 μM diethyldithiocarbamate, 50–500 μM *S*-mephenytoin and 2–20 μM quinidine did not markedly inhibit V11294 metabolism to either V10331, V10332 or V11689. In addition, V11294 metabolism to V10332 and V11689 was not markedly inhibited by 2–20 μM sulfaphenazole.

V11294 was metabolized by some of the cDNA-expressed human CYP forms examined. Using human B-lymphoblastoid cell microsomal preparations containing cDNA-expressed CYP3A4, V11294 was metabolized in a time-dependent manner to V10331, V10332, V11689 and V11690. V11294 was also metabolized to V10331 by cDNA-expressed CYP2C9 and CYP2D6, to V10332 by cDNA-expressed CYP2C9, CYP2C19 and CYP2D6 and to V11689 by cDNA-expressed CYP2C9. At extended incubation times, V11294 was also metabolized to V10331 by cDNA-expressed CYP2C19, to V10332 by cDNA-expressed CYP2A6, to V11689 by cDNA-expressed CYP2D6 and to V11690 by cDNA-expressed CYP2C9. In contrast, V11294 was not metabolized by control human B-lymphoblastoid cell microsomal preparations and microsomal preparations containing cDNA-expressed CYP1A2, CYP2B6, CYP2C8 and CYP2E1.

Although V11294 could be metabolized to some products by cDNA-expressed CYP2C9, CYP2C19 and CYP2D6, V11294 metabolism to any product was greatest with the cDNA-expressed CYP3A4 preparation (figure 3). Previous studies have demonstrated that levels of CYP2C19 and CYP2D6 are much lower than levels of CYP3A4 in human liver (Lecoeur *et al.* 1994, Shimada *et al.* 1994, Rodrigues 1999). Thus assuming these CYP forms have similar affinities (i.e. K_m values) for V11294 biotransformation, neither CYP2C19 nor CYP2D6 would have any major role in V11294 metabolism *in vivo*. In contrast, levels of CYP2C9 are relatively abundant in human liver (Lecoeur *et al.* 1994, Shimada *et al.* 1994, Rodrigues 1999), suggesting that this CYP form may have a minor role in hepatic V11294 metabolism.

In summary, the results of this study demonstrate that V11294 can be metabolized to V10331, V10332, V11689 and V11690 by human liver microsomal preparations. The three primary metabolites, namely V10331, V10332 and V11689, constitute major metabolites, whereas the secondary metabolite V11690, which can be formed from either V10332 or V11689, is only a minor

metabolite. By correlation analysis, chemical inhibition and the use of cDNA-expressed CYP isoforms, V11294 metabolism in human liver microsomes appears to be primarily catalysed by CYP3A forms.

Acknowledgements

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Requirement of additional adenylate cyclase activation for the inhibition of human eosinophil degranulation by phosphodiesterase IV inhibitors

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Abstract

Human eosinophils contain predominantly phosphodiesterase type IV, but selective inhibitors of this isoenzyme fail to inhibit certain eosinophil responses such as degranulation. In this study, the effect of activation of adenylate cyclase on the ability of several highly selective PDE IV inhibitors to inhibit complement C5a-induced O_2^- release and degranulation of human eosinophils in vitro was investigated. All four selective PDE IV inhibitors, *N*-(3,5-dichloropyrid-4-yl)-3-cyclopentyl-oxy-4-methoxybenzamide (RP 73401), rolipram, *N*-(3,5-dichloropyrid-4-yl)-[1-(4-fluorobenzyl)-5-hydroxy-indol-3-yl]glyoxylacidamide (AWD 12-281) and c-4-cyano-4-(3-cyclopentyloxy-4-methoxyphenyl-r-1-cyclohexane carboxylic acid) (SB 207499) potently inhibited C5a-induced O_2^- generation (IC_{50} = 0.03, 0.42, 0.55 and 0.86 μM, respectively), but generally failed to inhibit degranulation. The only exception was AWD 12-281, which inhibited degranulation (IC_{50} = 16.2 μM). In the presence of different AC activators (histamine, salbutamol, prostaglandin E_2 and forskolin), the PDE IV inhibitors became potent inhibitors of degranulation. The interaction between the PDE IV inhibitors and the AC activators resulted in a synergistic increase in intracellular levels of adenosine 3′, 5′-monophosphate (cAMP). These results show that PDE IV inhibitors generally require an additional cAMP signal to be able to inhibit eosinophil degranulation, and that this signal can be generated via both membrane receptors and direct AC activation. This may be relevant to the in vivo effectiveness of PDE IV inhibitors in eosinophilic inflammation. © 2001 Published by Elsevier Science B.V.

Keywords: Eosinophil; Degranulation; Phosphodiesterese IV inhibitors; cAMP; Adenylate cyclase; Synergism

1. Introduction

Adenosine 3′,5′-cyclic monophosphate (cAMP) is an important intracellular messenger that regulates many cellular activities. In many pro-inflammatory and immune cells, an increase in the intracellular concentration of this nucleotide generally leads to inhibition of responses (Dent et al., 1991; Fonteh et al., 1993; Prabhakar et al., 1994). For this reason, agents that elevate intracellular cAMP have potential as anti-inflammatory and anti-allergic drugs. Elevation of intracellular cAMP levels can be achieved through the activation of adenylate cyclase (adenylyl cyclase; EC 4.6.1.1) either directly or through appropriately coupled membrane receptors, as well as by preventing the hydrolysis of cAMP by the cyclic nucleotide phosphodiesterases (3′, 5′ cyclic nuclotide 5′-nucleotidohydrolase; EC 3.1.4.17).

PDEs represent a large number of isoenzymes which have been grouped into seven isoenzyme families (PDE 1 to PDE VII) based on the encoding gene or gene family as well as other criteria such as substrate specificity and kinetics, tissue distribution and sensitivity to inhibitors (Beavo, 1995; Conti et al., 1995; Loughney and Ferguson, 1996). Although many cell types may contain more than one PDE isoenzyme, PDE IV appears to be the predominant isoform in virtually all inflammatory cells (Torphy and Undem, 1991; Giembycz, 1992). Indeed, PDE IV inhibitors have been reported to be effective down-regulators of the various responses of pro-inflammatory cells, including eosinophils (Semmler et al., 1993; Torphy et al., 1994; Hatzelmann et al., 1995; Au et al., 1998).

In the pathophysiology of allergic disease, especially asthma, eosinophils are known to play important roles (Frigas and Gleich, 1986; Barnes, 1989). They infiltrate the asthmatic lung where they release tissue-damaging granular proteins such as the major basic protein and eosinophil peroxidase, lipid mediators such as the leuko-

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trienes, as well as oxygen-free radicals which, in concert, orchestrate bronchial inflammation and subsequent bronchial hyperresponsiveness (Laitinen et al., 1985; Motijima et al., 1989).

The promise of PDE IV inhibitors as effective anti-asthma drugs is based mainly on their anti-inflammatory effect (Schudt et al., 1995; Giembycz, 2000), and a major component of this effect is the ability to inhibit eosinophil responses. In guinea pigs, PDE IV inhibitors have been reported to inhibit the respiratory burst and degranulation of eosinophils (Dent et al., 1991; Souness et al., 1991; 1995) as well as their infiltration into allergen-challenged lungs (Underwood et al., 1993; Banner and Page 1995). Only few studies have addressed the effect of PDE inhibitors on human eosinophil function. While both non-selective PDE inhibitors such as theophylline and selective PDE IV inhibitors such as rolipram have been reported to inhibit the human eosinophil respiratory burst and chemotaxis (Dent et al., 1994; Cohan et al., 1996), only non-selective PDE inhibitors appear to be able to inhibit eosinophil degranulation (Hatzelmann et al., 1995; Ezeamuzie and Al-Hage, 1998). In their studies, Hatzelmann et al. (1995) have shown that in the presence of the β_2-adrenoceptor agonist, salbutamol, the selective PDE IV inhibitors became effective inhibitors of eosinophil degranulation. It was not determined, however, whether such potentiation was a consequence of a parallel elevation of intracellular cAMP or whether other methods of activating adenylate cyclase were equally effective in potentiating the inhibitory effect. The purpose of the present work was, therefore, to investigate the requirement for an additional cAMP signal by a number of PDE IV inhibitors *N*-(3,5-dichloropyrid-4-yl)-3-cyclopentyl-oxy-4-methoxybenzamide (RP 73401), rolipram, *N*-(3,5-dichloropyrid-4-yl)-[1-(4-fluorobenzyl)-5-hydroxy-indol-3-yl]glyoxylacidamide (AWD 12-281) and c-4-cyano-4-(3-cyclopentyloxy-4-methoxyphenyl-r-1-cyclohexane carboxylic acid) (SB 207499) for the inhibition of complement C5a-induced degranulation of human eosinophils in vitro. It also aimed at determining whether such a signal can be generated by other means of activating adenylate cyclase.

2. Materials and methods

2.1. Isolation of blood eosinophils

Fresh blood was obtained from consenting healthy adults or mildly atopic individuals who have no allergic disease. Eosinophils were purified by a slight modification of the immunomagnetic method (Hansel et al., 1991). Briefly, three parts of sodium citrate–anti-coagulated (13 mM final) blood was mixed with one part of 1% (w v^{-1} of 0.9% saline) hydrated methylcellulose solution to sediment the erythrocytes over 30 min at room temperature. The leucocyte-rich supernatant was collected and the leucocytes were recovered by centrifugation. After aspiration of the platelet-rich supernatant, the pelleted leucocytes were washed in "wash buffer" (Ca^{2+}- and Mg^{2+}-free, 10 mM HEPES-buffered Hanks balanced salt solution containing 0.25% bovine serum albumin) and resuspended in the same buffer at approximately 10% of the original blood volume. A 2-ml aliquot was then layered on a two-step Percoll gradient (1.080 and 1.090 g ml^{-1}) and centrifuged at $900 \times g$ on a Beckman (GS-6R) centrifuge for 20 min at room temperature. The upper layers (mononuclear cells and Percoll) were discarded and the pellet (granulocytes) was recovered and washed twice in the same buffer by centrifugation at $600 \times g$ for 10 min at 4°C. After hypotonic lysis of contaminating erythrocytes with ice-cold distilled water and readjustment of the tonicity with the same volume of double-strength saline, the cells were washed, counted and resuspended at a concentration of 2×10^7 cells ml^{-1} in the wash buffer. For eosinophil purification, 1.25 ml of the granulocyte suspension was then mixed with 5-μl mouse anti-human CD16 monoclonal antibody in a siliconized test tube, and incubated on ice for 1 h with frequent gentle rotation. Cells were then washed twice in wash buffer and then resuspended in 500 μl of prewashed immunomagnetic beads, pre-coated with affinity-purified sheep anti-mouse immunoglobulin G (2×10^8 coated beads), and incubated in ice for 1 h with frequent tube rotation. The immunomagnetically labeled neutrophils were removed by magnetic extraction. The purified eosinophils were then recovered by centrifugation and resuspended in reaction buffer ("wash buffer" containing 2 mM Ca^{2+} and 1 mM Mg^{2+}) for experiments. The eosinophil purity was assessed by differential counting of a Wright–Giemsa-stained cytosmear. The final cell preparation routinely consisted of over 98% pure eosinophils. Viability was determined by Trypan blue exclusion and always exceeded 98%.

2.2. Superoxide anions release

Superoxide anion (O_2^-) generation was determined by the superoxide dismutase-inhibitable reduction of ferricytochrome *c* (Sedgwick et al., 1988). Purified eosinophils were resuspended at a concentration of 5×10^5 cells ml^{-1}, and 50-μl aliquots were dispensed into each well of a 96-well microplate containing 50 μl of cytochrome *c* (100 μM, final) and 50-μl reaction buffer. After pre-warming of the mixture, 50 μl of the stimulus was then added and the mixture was incubated at 37°C for 1 h. Corresponding wells containing 0.6 μM (final) SOD were included to assess specific O_2^- formation. In experiments in which the effect of drugs was assessed, 50 μl of the drugs was added in place of the buffer and incubated with the cells for 10 min before the addition of the stimulus. After incubation, 150 μl was transferred to a fresh plate and the absorbance was read at 550 nm on a Titertek Multiscan (Flow Labs, Rickmansworth, UK) plate reader. The amount of O_2^- generated was estimated as nmol ferricytochrome *c* re-

duced/10^6 cells h^{-1}, using an extinction coefficient of 2.1×10^{-4} M^{-1} cm^{-1}.

2.3. Eosinophil peroxidase release

Fifty microlitres of pre-warmed cell suspension containing 2.5×10^4 cells was dispensed into each well of a microplate. Then, 100 μl of the reaction buffer containing 10 μg ml^{-1} cytochalasin B was added and, after a 10-min pre-incubation, the cells were stimulated with 50 μl of human recombinant C5a. The mixture was further incubated for 30 min at 37°C. It had been determined in pilot experiments that this time was sufficient for the virtual completion of the degranulation process. At the end of the incubation period, the reaction was stopped by cooling on ice and after centrifugation at $600 \times g$ for 10 min, 50-μl aliquots of the supernatant as well as Triton X-100-lysed cells (for total content determination) were taken for the determination of the released enzymes. EPO activity was measured by the O-phenylenediamine method as previously reported (Kroegel et al., 1989). Briefly, OPD substrate solution, containing 0.4 mg ml^{-1} OPD and 0.4 mg ml^{-1} urea hydrogen peroxide in phosphate buffered saline-citrate buffer (pH 4.5), was prepared from SIGMA FAST® OPD tablets. One hundred microlitres of this substrate was added to 50 μl of the samples in a microplate and incubated for 30 min at 37°C. After incubation, the reaction was then stopped with 50 μl of 4 M H_2SO_4 and the absorbance was read at 490 nm. Values are expressed as percentages of the total content, using the amount obtained in half the same number of cells, after lysis, as 50%. The recovery of released EPO activity was usually above 80% at the end of a 30-min incubation.

2.4. Intracellular cyclic AMP determination

One million purified eosinophils, resuspended in 100 μl of BSA-free reaction buffer, were dispensed into each well of a 96-well plate containing 50 μl of the test PDE inhibitor or vehicle and incubated for 10 min at 37°C. The reaction was then started by the addition of 50 μl of warmed stimuli (AC activators). Three minutes later—a time previously determined to be best for this response (10 min for forskolin)—the reaction was stopped by the direct addition of 22.2 μl of 1N HCl. After thorough mixing of the reaction mixture and further incubation for 10 min, the plate was centrifuged at $1500 \times g$ for 10 min and 200 μl of the supernatant was taken and stored at −43°C, pending cAMP assay.

Cyclic AMP levels were measured, after acetylation, using a commercially available enzyme-linked immunosorbent assay kit, and following the manufacturer's instructions. The sensitivity of the assay was 0.01 pmol/well.

2.5. Drugs and chemical reagents

The following drugs and reagents were obtained from Sigma, St Louis, MO, USA: recombinant human C5a, Percoll, HEPES buffer, BSA, OPD, dimethylsulphoxide (DMSO), 3-isobutyl-1-methylxanthine (IBMX), theophylline, histamine dihydrochloride, prostaglandin E_2, cytochalasin B and all inorganic salts. Other drugs were obtained as follows: AWD 12-281 (Arzneimittelwerk Dresden, Radebuel, Germany); RP 73401 (Rhone-Poulenc Rorer, Dagenham, UK); SB 207499 (SmithKline Beecham, King of Prussia, PA, USA); rolipram and salbutamol hemisulphate (Research Biochemicals, Natick, MA, USA) and mouse monoclonal anti-human CD16 antibody (clone FcR gran1) (CLB, Amsterdam, Netherlands. The magnetic beads (coated with sheep anti-mouse immunoglobulin G) were supplied by Dynal, Oslo, Norway, while the cAMP assay kit (direct method) was obtained from Assay Designs, Ann Arbor, MI, USA.

Stock solutions of PDE IV inhibitors were made in DMSO to concentrations in the range ($1–4 \times 10^{-1}$ M) and then diluted directly in buffer. Prostaglandin E_2 and forskolin were initially dissolved in ethanol while salbutamol was dissolved in 0.1 N HCl. The final concentration of the solvents at the highest drug concentrations did not exceed 0.05%—a concentration that has no effect on eosinophil responses. All the other drugs and reagents were first dissolved in distilled water and diluted in reaction buffer.

2.6. Statistical analysis

Experimental data are presented as means ± S.D. from the number (n) of independent experiments. The concentrations producing 50% inhibition (IC_{50} values) were calculated from the concentration–effect curves by non-linear regression analysis using Graph*h*Pad InPlot (GraphPad Software, Philadelphia, USA). Statistical significance (P) was determined by the unpaired t-test or by one-sample t-test as appropriate (InStat, Graph*h*Pad, Software, USA).

3. Results

3.1. Effect of selective and non-selective PDE inhibitors on O_2^- release and degranulation

In the absence of CB, C5a induced a pronounced and concentration-dependent release of O_2^- from purified eosinophils (data not shown). At the sub-optimal concentration of 10 nM, O_2^- release was equivalent to that reducing 45.8 ± 6.7 nmol cytochrome $c/10^6$ cells h^{-1}.

Pre-incubation of eosinophils with the 4 PDE IV inhibitors resulted in a potent and concentration-dependent inhibition of O_2^- release by all the agents (Fig. 1A). The

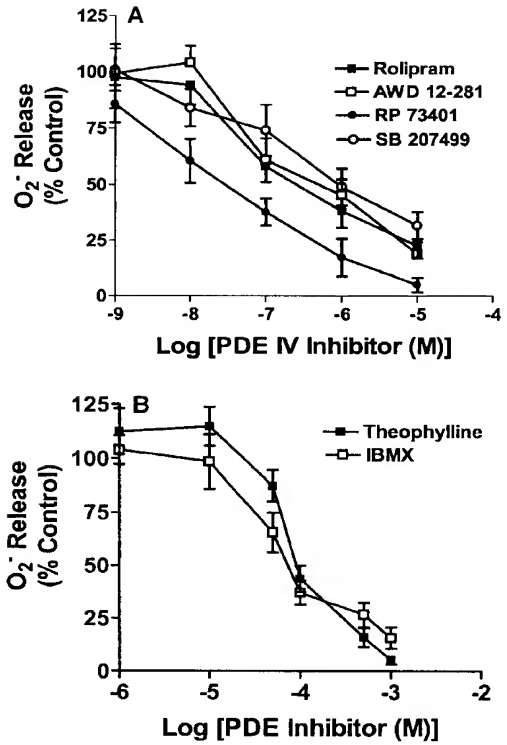

Fig. 1. The effect of the selective PDE IV inhibitors (A) and the non-selective PDE inhibitors (B) on O_2^- release from human eosinophils induced by C5a (10 nM). The uninhibited (control) release was equivalent to the amount reducing 45.8 ± 6.7 nmol cytochrome $c/10^6$ cells h^{-1}. Cells were incubated with the drugs for 10 min before stimulation. Values are means $\pm$ S.D., $n = 5$.

IC_{50} values (95% CI) were 0.03 (0.01–0.06) μM, 0.42 (0.22–0.70) μM, 0.55 (0.28–0.85) μM and 0.86 (0.48–1.36) μM, for RP 7341, rolipram, AWD 12-281 and SB 207499, respectively, $n = 5$. The non-selective PDE inhibitors theophylline and 3-isobutyl-1-methylxanthine (IBMX) were also effective, but the potency was considerably less (Fig. 1B)—the IC_{50} values (95% CI) being 78.4 (40.0–106.3) μM and 93.7 (62.2–126.8) μM, respectively, $n = 5$. At the concentrations tested, none of the PDE inhibitors had any significant oxygen radical-scavenging effect, as determined with the hypoxanthine–xanthine oxidase system.

In the presence of CB (5 μg ml^{-1}), the sub-optimal concentration of C5a (10 nM) induced the release of 13–45% of the total cell EPO content. In contrast to the effect of the PDE IV inhibitors on O_2^- release, pre-treatment of cells with these drugs was generally ineffective in inhibiting EPO release (Fig. 2A). The only exception was AWD 12-281, which produced a concentration-dependent inhibition that was statistically significant at concentrations above 1 μM. Its IC_{50} value (95% confidence interval) was 16.3 (9.3–22.8) μM, $n = 7$, and at 50 μM, the inhibition was ≈ 65%.

Unlike most of the selective PDE IV inhibitors, the non-selective PDE inhibitors, theophylline and IBMX, effectively inhibited EPO release (Fig. 2B). Their IC_{50} values (95% CI) were 246 (158.4–335.3) μM and 115.8 (78.3–142.6) μM, respectively, $n = 4$. At 1 mM, both drugs achieved almost complete abolition of degranulation. None of the inhibitors induced direct EPO release or affected EPO measurements.

3.2. The effect of activators of adenylate cyclase on the inhibition of EPO release by PDE inhibitors

To study the effect of the AC activators on the ability of PDE inhibitors to inhibit EPO release, the effects of these agents alone were first determined. As shown in Fig. 3, concentrations of salbutamol and forskolin up to 10 μM had no effect on EPO release. However, histamine and, to a lesser extent, prostaglandin E_2 were effective in inhibiting EPO release. The IC_{50} value (95% CI) for histamine was 1.5 (0.8–2.8) μM, while the inhibition by prostaglandin E_2, though statistically significant at 1–10 μM,

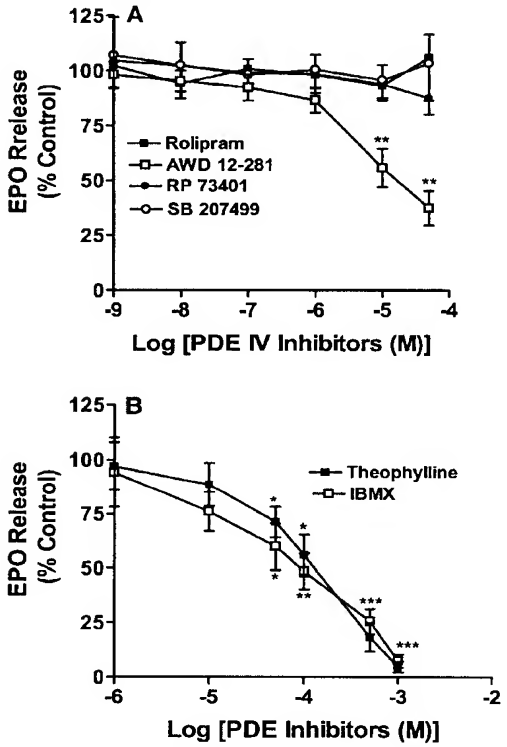

Fig. 2. The effect of the selective PDE IV inhibitors (A) and the non-selective inhibitors (B) on EPO release from human eosinophils induced by C5a (10 nM) in the presence of 5 μg ml^{-1} cytochalasin B. The uninhibited release was in the range 13–45% of the total cell content. Cells were incubated with the drugs for 10 min before stimulation. Values are means $\pm$ S.D., $n = 7$ for a, and $n = 4$ for b. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

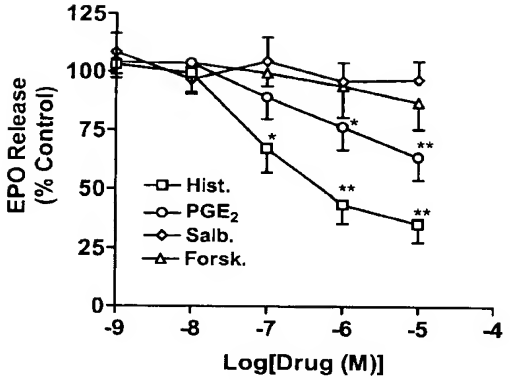

Fig. 3. The effect of adenylate cyclase activators (histamine, Prostaglandin E_2, salbutamol and forskolin) on EPO release from human eosinophils induced by C5a (10 nM) in the presence of 5 μg ml^{-1} cytochalasin B. The uninhibited release was in the range 13–45% of the total cell content. Cells were incubated with the drugs for 10 min before stimulation. Values are means ± S.D., $n = 5$. $^{*}P < 0.05$, $^{**}P < 0.01$.

did not reach 50%. Using concentrations of these activators that had little or no effect of their own, the interaction with the PDE inhibitors on the inhibition of EPO release was subsequently studied. Rolipram and AWD 12-281 were used as prototypes of inactive and active PDE IV inhibitors, respectively, while theophylline was used as a prototype of a non-selective PDE inhibitor. As shown in Fig. 4A, in the presence of histamine (0.03 μM), prostaglandin E_2 (0.1 μM), salbutamol (1 μM) and forskolin (10 μM), the completely inactive rolipram became a highly potent inhibitor. Histamine and prostaglandin E_2 were the most effective in potentiating the rolipram effect, followed by salbutamol and lastly, forskolin. For example, the percentage inhibitions produced by histamine, prostaglandin E_2, salbutamol and forskolin alone at the above given concentrations were 17.3%, 11.4%, 9.7% and 8.7%, respectively, while the inhibition produced by rolipram (10 μM) alone was 1.8%. Together, the percentage inhibition (expected sum of the individual effects) was 64.0 (19.1)%, 58.8 (13.2)%, 54.2 (11.5)% and 42.6 (10.5)%, respectively. The combined effects were all significantly greater than the expected sum of the individual effects, $P < 0.05$–0.001, $n = 7$, thus indicating strong synergism.

Similar effects were seen with AWD 12-281 (Fig. 4B), but in this case prostaglandin E_2 was the most effective potentiator, followed by histamine. Again, the potentiating effect of forskolin was the weakest of the four AC activators—its interaction being essentially additive in this case. Interestingly, the synergistic interaction of AWD 12-281 with histamine, prostaglandin E_2 and salbutamol occurred even at concentrations of AWD 12-281 that produced a significant inhibition on their own. For theophylline, the interaction with all the AC activators was essentially additive at lower concentrations, but less than additive at higher concentrations (Fig. 4C).

3.3. Interaction between PDE inhibitors and AC activators on intracellular cAMP responses

Table 1 shows the results of the interaction between the PDE inhibitors and the AC activators on the intracellular cAMP response. In the absence of PDE inhibitors, the intracellular cAMP level in unstimulated cells was only 0.15 ± 0.20 pmol cAMP/10^6 cells, $n = 5$. Tested alone, none of the AC activators at the concentrations tested, histamine (30 μM), salbutamol (10 μM) and forskolin (10 μM), produced a significant net increase in intracellular cAMP levels, $n = 4$. In the absence of an AC activator, the

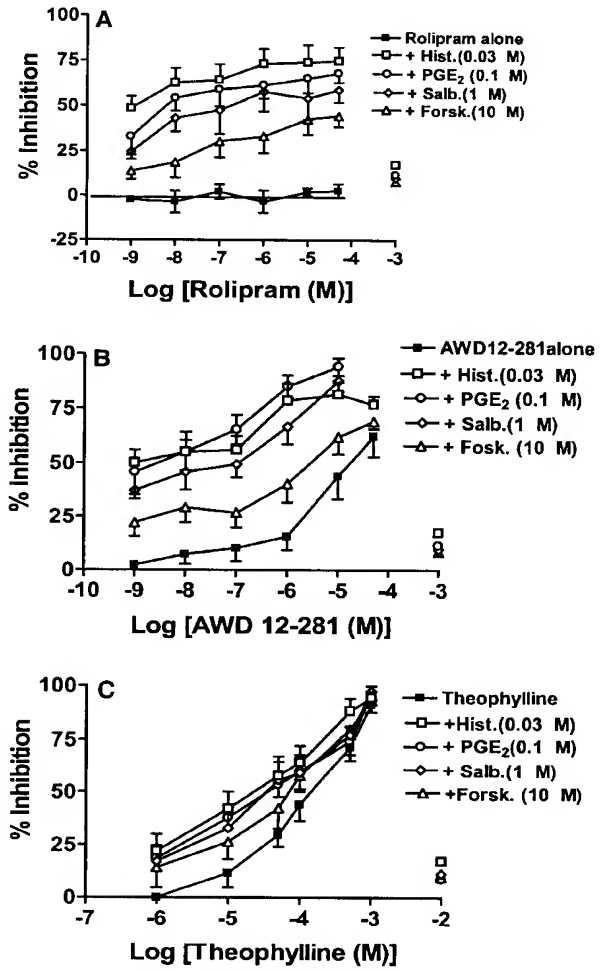

Fig. 4. The effect of the adenylate cyclase (AC) activators (histamine, prostaglandin E_2, salbutamol and forskolin) on the ability of rolipram (A), AWD 12-281 (B) and theophylline (C) to inhibit EPO release from human eosinophils induced by complement fragment C5a (10 nM). The uninhibited release was in the range 13 to 45% of the total cell content. Cells were first pre-incubated with the PDE inhibitors for 10 min, followed by the AC activators for another 10 min before stimulation. Values are means ± S.D., $n = 6$ or 7. The unconnected symbols represent the effect of the AC activators alone.

Table 1
The interaction of PDE inhibitors and adenylate cyclase activators in the elevation of intracellular cAMP in human eosinophils
ND = not done.

	Net increase in intracellular cAMP (pmol/10^6 eosinophils)			
	+ Buffer, $n = 5$	+ Histamine (30 μM), $n = 4$	+ Salbutamol (10 μM), $n = 4$	+ Forskolin (10 μM), $n = 4$
Buffer	[0.15 ± 0.20]	0.10 ± 0.15	0.06 ± 0.10	0.16 ± 0.21
Rolipram (10 μM)	0.24 ± 0.17	1.46 ± 0.38[a]	0.62 ± 0.29	1.77 ± 0.30[a]
Rolipram (30 μM)	0.48 ± 0.27[b]	2.00 ± 0.33[a]	1.54 ± 0.40[a]	2.63 ± 0.45[c]
AWD 12–218 (10 μM)	0.25 ± 0.10	1.60 ± 0.44[a]	1.20 ± 0.35[a]	1.56 ± 0.37[a]
AWD 12–281 (30 μM)	0.40 ± 0.21[b]	1.73 ± 0.27[a]	1.67 ± 0.38[a]	2.36 ± 0.48[a]
Theophylline (300 μM)	0.15 ± 0.24	0.88 ± 0.36[a]	ND	1.06 ± 0.24[a]
Theophylline (1000 μM)	0.32 ± 0.18	2.12 ± 0.48[a]	ND	ND

[a] $P < 0.05$ vs. the expected sum of values for PDE inhibitor alone and AC alone.
[b] $P < 0.05$ vs. buffer alone.
[c] $P < 0.01$ vs. the expected sum of values for PDE inhibitor alone and AC activator alone.

PDE inhibitors produced small dose-dependent increases in cAMP that reached statistical significance only at the higher concentrations of rolipram (30 μM) and AWD 12-281 (30 μM). However, when combined with the AC activators, synergistic increases in cAMP responses were seen for both the selective PDE IV inhibitors—rolipram and AWD 12-281—and the non-selective PDE inhibitor—theophylline. For example, in the absence of an AC activator, rolipram (30 μM) produced a net increase of 0.48 ± 0.27 pmol cAMP/10^6 cells, whereas in the absence of rolipram, the net increase produced by the AC activators was 0.10 ± 0.15, 0.06 ± 0.10 and 0.16 ± 0.21 pmol cAMP/10^6 for histamine (30 μM), salbutamol (10 μM), and forskolin (10 μM), respectively. When rolipram was combined with these AC activators, the net increase was 2.00 ± 0.33, 1.54 ± 0.40 and 2.63 ± 0.45 pmol/10^6 cells, respectively. The differences between these values and the expected sums of the individual values for rolipram and the corresponding AC activators were statistically significant, $P < 0.05$–0.01, thus indicating synergism.

It is to be noted that, in the absence of an AC activator, even the highest concentration of theophylline (1000 μM) (which inhibited EPO release by > 90%) failed to produce a significant increase in intracellular cAMP.

4. Discussion

In this study, four highly potent and selective PDE IV inhibitors [rolipram, AWD 12-281, RP 73401 and SB 207499) were used to further investigate the differential effect of selective and non-selective PDE inhibitors, with special reference to human eosinophil degranulation and the role of intracellular cAMP. The results demonstrated that while all four PDE IV inhibitors were effective in inhibiting C5a-induced O_2^- release, none, except AWD 12-281, had any effect on EPO release. In contrast, the non-selective inhibitors, theophylline and IBMX were effective in inhibiting both responses. For the inhibition of O_2^- release, the PDE IV inhibitors were, however, two to three orders of magnitude more potent than the non-selective ones, though with comparable efficacy. These results are in agreement with those previously reported for the respiratory burst in eosinophils of both guinea pigs (Dent et al., 1991; Souness et al., 1991, 1995) and humans (Cohan et al., 1996; Dent et al., 1994; Ezeamuzie and Al-Hage, 1998) as well as for degranulation of human eosinophils (Hatzelmann et al., 1995). In human whole blood, rolipram was reported to inhibit platelet activating factor-induced up-regulation of adhesion molecules in eosinophils (Berends et al., 1997). This agrees with the present result with respect to O_2^- release but not degranulation, and thus supports the view that different responses of eosinophils can be differentially affected by drugs (Ezeamuzie and Nwankwoala, 1997).

The combined inhibitory effect of PDE IV inhibitors and the AC activators on eosinophil degranulation was mostly synergistic, and this was reflected in the corresponding synergistic increase in the intracellular levels of cAMP. For theophylline, however, there was a synergistic increase in intracellular cAMP, but only a marginal increase in the inhibition of degranulation (additive at most). This may suggest that the non-selective PDE inhibitors generally require lower intracellular levels of cAMP compared with selective ones, in order to attenuate this response or that the non-selective inhibitors are somehow able to inhibit degranulation in an essentially cAMP-independent manner.

The finding that AWD 12-281 can inhibit eosinophil degranulation is quite interesting, and perhaps makes the drug the first PDE IV inhibitor to possess this property. It is unlikely that this unique effect of AWD 12-281 is related to its potency since the drug is comparable in potency to rolipram and SB 207499 and at least 10 times

less potent than RP 73401, in both cell-free PDE IV isoenzyme inhibition and inhibition of pro-inflammatory cell responses (Cohan et al., 1996; Barnette et al., 1998; Heer et al.,1999). Furthermore, the fact that AWD 12-281, synergized with AC activators, causes inhibition of degranulation even at concentrations of AWD 12-281 that were effective on their own, suggests that the inhibitory activity may be unrelated to the PDE IV inhibitory activity of the drug.

In addressing the reason why the non-selective PDE inhibitors, but not the selective PDE inhibitors, inhibited eosinophil degranulation, Hatzelmann et al. (1995) showed that rolipram became an effective inhibitor in the presence of the β_2-adrenoceptor agonist salbutamol. A similar interaction was also reported for the inhibition of zymosan-induced interleukin-8 release from human neutrophils (Au et al., 1998) and O_2^- release from human eosinophils (Dent et al., 1994). These observations would suggest that selective PDE IV inhibitors might require an additional cAMP signal to be effective. The present study confirms such findings and demonstrates that such signals could be generated via various AC-linked receptors (histamine H_2-receptors, prostaglandin E receptors and β_2-adrenoceptors), as well as via direct AC activation (forskolin). Furthermore, by actually measuring the cAMP response elicited by the PDE IV inhibitors alone and in combination with histamine, salbutamol or forskolin, the present study has provided direct evidence that synergistic cAMP generation was indeed the additional signal required. The limitation imposed by the large number of eosinophils required in these experiments did not allow us to test prostaglandin E_2.

Why the non-selective PDE inhibitors do not require this extra cAMP signal to inhibit degranulation is unclear. It is unlikely that the inhibition of more than one PDE isoenzyme is necessary for degranulation to be attenuated. This is because the human eosinophil is known to contain predominantly the PDE IV isoenzyme (Torphy and Undem, 1991; Giembycz, 1992), and also because the highly selective PDE IV inhibitor AWD 12-281 was effective. One possibility is that the non-selective PDE inhibitors may, in addition to PDE inhibition, also somehow activate AC or directly activate some cAMP-dependent protein kinases, as has been previously suggested (Parsons et al., 1988; Tomes et al., 1993).

Although the synergistic generation of extra cAMP appears to be the basis for the anti-degranulatory effectiveness of PDE IV inhibitors in the presence of AC activators, a closer look at the relationship between the level of intracellular cAMP generated and the extent of inhibition revealed a number of inconsistencies. Firstly, forskolin (10 μM), which synergized relatively more effectively with rolipram than the receptor-mediated AC activators in raising intracellular cAMP, was the least effective in potentiating the anti-degranulatory effect of rolipram. Secondly, at the concentration of 30 μM, both rolipram and AWD 12-281 produced comparable cAMP responses, whereas only AWD 12-281 was effective in inhibiting degranulation in the absence of AC activators. Thirdly, in the absence of PDE inhibitors, histamine and, to a lesser extent, prostaglandin E_2 (at concentrations above 0.1 μM) were able to inhibit eosinophil degranulation, but under the same conditions, they were incapable of significantly elevating intracellular cAMP.

The reason for these discrepancies is uncertain, but it highlights the complex nature of the role of cAMP in the regulation of eosinophil responses. There is, of course, the possibility that cAMP generated by different agents or under different circumstances may be qualitatively different or differentially used by different sub-populations of cAMP-dependent protein kinases. No evidence is yet available to support these possibilities. Further studies are on-going to further clarify the exact role of cAMP in the regulation of eosinophil functions.

The PDE IV inhibitors used in this study are all experimental drugs for which therapeutic concentrations have yet to be established. However, given that the synergistic interactions of these compounds with AC activators occurred even at very low concentrations of the PDE inhibitors (as low as 1 nM), it is likely that such interactions will be therapeutically relevant. For theophylline, the essentially additive interaction with the AC activators was also quite apparent in its therapeutic concentration range of 10–100 μM. These interactions are, therefore, expected to contribute to the overall anti-inflammatory potency of these drugs in vivo. For example, in the bronchial inflammation that underlines asthma, the concentrations of the endogenous AC-activating autocoids, such as histamine and prostaglandin E_2, are bound to be raised. In such a scenario, the resulting potentiation of the action of PDE IV inhibitors might in fact be a major contributing factor to the anti-inflammatory and anti-asthma potency of these drugs.

In summary, this study has shown that, unlike the non-selective PDE inhibitors, selective PDE IV inhibitors require an additional cAMP signal to be able to inhibit eosinophil degranulation. This signal can be generated by both direct and receptor-mediated AC activation. The exact cause–effect relationship between intracellular cAMP and inhibition of degranulation is, however, not a simple one, hence, more studies are required to clarify the role of cAMP in the regulation of human eosinophil responses. Nevertheless, the potentiation of the inhibitory action of PDE IV inhibitors by endogenous autacoids is likely to be important in the overall in vivo anti-inflammatory action of these drugs.

Acknowledgements

The excellent technical assistance of Mrs. Elizabeth Philips is greatly appreciated.

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AWD-12-281 (inhaled), elbion/GlaxoSmithKline

Company	**ASTA Medica AG**
Highest Dev Status	Discontinued
Indications	Chronic obstructive pulmonary disease Asthma
Actions	Anti-inflammatory PDE 4 inhibitor Bronchodilator
Technologies	Powder formulation, inhalant

Reason for update on 11 July 2006

one or more development status entries have been updated, 1 reference added [**677764**]

AWD 12-281-2

Summary

GlaxoSmithKline (GSK), under license from **elbion** (formerly ASTA Medica), was developing an inhaled formulation inhaled formulation of the 5-hydroxyindole PDE 4 inhibitor AWD-12-281 (GW-842470; 842470) for the potential treatment of chronic obstructive pulmonary disease (COPD) [**457611**]. The compound was initially in development for allergic rhinitis, as a **nasal formulation** (qv), and for asthma, which reached phase II clinical trials [**467734**]. However, by December 2003, these indications had been dropped [**515870**]. By May 2005, phase II trials for COPD were ongoing [**612889**], however, in February 2006, GSK discontinued its part in development due to poor efficacy [**676949**].

GSK, under license from elbion, is also developing a **topical cream formulation (qv) of AWD-12-281** for the potential treatment of atopic dermatitis.

By June 2002, development of an orally active analog of AWD-12-281, AWD-12-343, had been discontinued [**456295**], [**457936**].

CLINICAL DATA

In December 2003, GSK expected to submit regulatory filings in 2007 [**516398**].

By October 2002, inhaled AWD-12-281 was in phase II trials for asthma and COPD [**467734**]; the phase II trials in COPD were ongoing in May 2005 [**612889**].

In November 2004, phase I data on AWD-12-281 in the treatment of COPD were presented at the Second SRI Phosphodiesterases in Drug Discovery and Development conference in Philadelphia, PA. No difference in bioavailability was reported between smokers and non-smokers, and there was no potentiation of rescue medication-induced cardiac adverse events. AWD-12-281 was safe and well tolerated [**571646**], [**612977**].

PRECLINICAL DATA

In November 2004, preclinical data on AWD-12-281 were presented at the Second SRI Phosphodiesterases in Drug Discovery and Development conference in Philadelphia, PA. AWD-12-281 was found to inhibit allergen-induced release of TNFalpha and pro-inflammatory cytokines. Allergen-induced eosinophil infiltration was inhibited in rats for up to 18 h. AWD-12-281 was found to be as active as beclomethasone in pig and ferret models of lipopolysaccharide (LPS)-induced neutrophil infiltration, and there was no evidence of emesis at high doses in ferrets and dogs. Bioavailability was high in rats and dogs following intratracheal administration, but very low when administered orally [**571646**].

In May 2001, data on AWD-12-281 were presented at the 97th American Thoracic Society meeting in San Francisco, CA. The secretion of serous mucus in mouse trachea was increased by AWD-12-281, which also reduced LPS-induced neutrophilia in rats and pigs [**412484**], [**467423**].

Preclinical data published between January and March 1999, supported the therapeutic value of AWD-12-281 for allergic asthma because of its strong anti-inflammatory properties and low emetic potential [**314134**]. In rodent models, the drug attenuated early- and late-phase bronchoconstriction and pulmonary eosinophilia [**314134**]. In order to overcome the dose-limiting nausea associated with oral administration of this class of drug, an aerosolized form of AWD-12-281 was tested [**318120**].

Between 1998 and 1999 a number of in vitro and in vivo studies demonstrated AWD-12-281 was a potent PDE inhibitor, abrogated LPS-induced secretion of pro-inflammatory cytokines and decreased levels of histamine-induced intracellular calcium. The compound also had a reduced emetic effect compared with

rolipram and **RP-73401** (qv) [**298817**], [**298703**], [**334829**], [**335293**].

OTHER ANALOGS
Data presented in June 2000 at the 27th National Medicinal Chemistry Symposium in Kansas City, MO, described the synthesis of a new orally active PDE4 inhibitor (AWD-12-343), based on the structure of AWD-12-281. Both AWD-12-281 and AWD-12-343 inhibited TNFalpha and GM-CSF secretion from human cells involved in allergic inflammatory responses. AWD-12-343 was also found to reduce LPS-induced lung neutrophilia in rats, and at this time, the compound had been selected for further investigation [**372205**], [**372149**]. By June 2002, however, elbion stated that the development AWD-12-343 had been discontinued [**456295**].

ADDITIONAL INFORMATION
In July 2002, elbion formed an agreement with GSK granting exclusive worldwide development, registration, manufacturing and commercialization rights to AWD-12-281 and other related compounds. GSK would be responsible for all development costs [**457611**].

For an expert analysis of the development of this drug, as of October 2005, please refer to the Literature Evaluation.

Development Status

HISTORY

Detailed status for **elbion AG**

Indication	Country	Status	Reference	Date
Asthma	Germany	Discontinued	**457611**	10 July 2002
Chronic obstructive pulmonary disease	Germany	Discontinued	**457611**	10 July 2002

Detailed status for **GlaxoSmithKline plc**

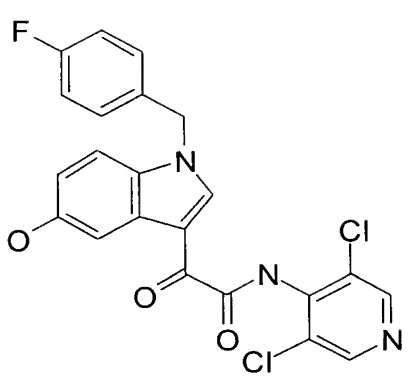
Indication	Country	Status	Reference	Date
Asthma	Germany	Discontinued	**515870**	03 December 2003
Chronic obstructive pulmonary disease	Germany	Discontinued	**676949**	01 February 2006

Licensing

Summary	Reference
elbion AG	
In July 2002, **GlaxoSmithKline** (GSK) and **elbion** signed a worldwide agreement to collaborate on the development and commercialization of **AWD-12-281** and associated backup compounds. GSK obtained exclusive worldwide development, registration, manufacturing and commercialization rights for **AWD-12-281** and would assume all responsibilities and costs. elbion received an upfront payment and was eligible to receive clinical and regulatory milestone payments depending on the progress of the various indications. elbion was also to receive royalties based on total worldwide annual net sales for all indications [**457611**]. In February 2006, GSK discontinued development due to poor efficacy [**676949**].	**457611**

Chemistry

Structure



Confidence Level : 1

Compound names associated with this drug

Name	Type
842470	Research Code
AWD-12-343	Research Code, Analogue
GW-842470	Research Code
AWD-12-281 (COPD), elbion/GlaxoSmithKline	
AWD-12-281 (inhaled), elbion/GlaxoSmithKline	
AWD-12-281 (asthma), elbion/GlaxoSmithKline	
AWD-12-281	Research Code
257892-33-4	CAS RN

Literature evaluation

Hans-Jurgen Gutke, Jan-Hinrich Guse, Moussa Khobzaoui, Thejavathi Renukappa-Gutke and Michael Burnet, **Synovo GmBH**, Tubingen, Germany

Submission date: 9 August 2005
Publication date: 18 October 2005

Introduction

AWD-12-281 is an inhibitor of phosphodiesterase (PDE)4, which is currently in development as an inhaled formulation for chronic obstructive pulmonary disease (COPD) and as a topical cream for atopic dermatitis and psoriasis. An intranasal formulation was previously in development for allergic rhinitis, however, this has now been discontinued. The compound was under sole development by **elbion AG** and has since been subject to a collaborative development agreement with **GlaxoSmithKline plc** (GSK) for which it has the code number GW-842470. AWD-12-281 differs from other compounds in this class in so far as it has been selected for its low systemic availability from topical application, with the objective of widening its therapeutic index. elbion has two other PDE4 inhibitors in preclinical development: **ELB-353** is an orally active non-hydroxylated aza-indole analog of AWD-12-281, and **ELB-526** has an undisclosed structure.

PDE4 is ubiquitous to inflammatory and immunomodulatory cells [**457183**], and the specific functions of these cells are broadly inhibited by selective PDE4 inhibitors [**304539**], [**402539**]. As PDE4 is the major cAMP-metabolizing enzyme in immune and inflammatory cells [**30771**], and an increase in the intracellular concentrations of cAMP leads to the inhibition of various inflammatory responses in many pro-inflammatory and immune cells [**417192**], it is likely that many of the effects of PDE4 inhibitors are due to the inhibition of cAMP hydrolysis [**402539**]. The strong general interest in PDE4 derives from initial observations of the activity and pharmacology of the non-selective PDE4 inhibitor theophylline and the PDE4-selective inhibitor **rolipram** [**480517**]. The combined effects of theophylline on cytokine production (suppression of TNF-alpha and upregulation of interleukin (IL)-10) and bronchodilation provided the initial suggestion that compounds in this class may have application in asthma and other respiratory diseases. However, small molecule interaction with PDE4 also results in mechanism related emesis. Despite large-scale efforts to separate emetic effects from anti-inflammatory effects, not one rationale has proved adequate to guide the synthesis of selective inhibitors [**266561**], [**276922**], [**381250**], [**588331**], [**592024**].

The apparent intractability of separating emesis from anti-inflammatory effects at the level of target selectivity has led to attempts to modify the distribution of the compounds in vivo, initially by selecting

properties incompatible with central nervous system (CNS) uptake and later through topical formulation. Unfortunately, modification of CNS permeation appears not to modulate emetogenic effects in various PDE4 inhibitor series [**480517**], and it is likely that the interacting emetic receptors exist both in the gastrointestinal tract and the periphery.

Against this background, an alternative approach to increase therapeutic index has been to formulate for topical (including inhaled) administration. AWD-12-281 has been optimized for this route of application and has, as a consequence, very low bioavailability via the oral route and high activity via inhalation [**612977**]. Systemic exposure is limited by retention of the compound at the site of application and by metabolic lability [**612977**].

In this respect, the therapeutic philosophy driving AWD-12-281 is one that resembles that used with corticosteroids, namely the design of compounds with either retention in the lung, or significant chemical instability such that they are rapidly deactivated in the systemic circulation.

An example of a product of the former strategy is **ciclesonide** [**213439**], a glucocorticoid prodrug developed by **Altana AG**, **Sanofi-Aventis** and **Teijin Pharma Ltd**. **Ciclesonide** is a hydrophobic prodrug that is retained in the lung where it is hydrolyzed to desisobutyryl-**ciclesonide**. Both the prodrug and the active form are subject to rapid first-pass metabolism leading to very low systemic exposure to the compound [**622553**]. Similarly, this approach has been taken by **PLIVA dd** with their so-called sterolides, which are conjugates of steroids and macrocycles with very low systemic availability, primarily due to high retention of the large hydrophobic compound at the site of application [**562362**], [**563994**]. Exploitation of metabolic instability as a means of limiting systemic effect is best illustrated by budesonide (see eg, reference [**625264**]).

AWD-12-281 is retained with a long half-life in the lung following inhalation. The compound enters the systemic circulation with bi-phasic kinetics, after which it is readily glucuronidated, resulting in limited systemic exposure and a plasma half-life of approximately 3 h [**612977**]. Any compound that is swallowed during inhalation is relatively poorly available (~ 3 %) and so contributes little to the systemic load [**612977**]. The combination of these properties ensures that systemic levels of the drug are low in studies performed in animal models.

In this review, the reference PDE4 inhibitor for AWD-12-281 will be **roflumilast** (**Altana AG/Tanabe Seiyaku Co Ltd**), which is more developmentally advanced. The positioning of **roflumilast** for oral administration is critical when comparing the two. The involvement of GSK in the development of AWD-12-281 will presumably be conducted in the context of both this experience with and the commercial future of their other advanced PDE4 inhibitor, **cilomilast** [**488868**]. The latter is still in development for COPD, as is AWD-12-281. Neither is being developed for asthma, perhaps reflecting the experience GSK have with the mode of action of PDE4 with regard to that indication, and the continued success of inhaled corticosteroids. However, COPD remains a major market without suitable therapy, and the underlying inflammation appears relevant to the mode of action of PDE4 inhibitors [**588333**]. Should the necessary doses prove to be well tolerated in humans, there is significant potential for this class of drugs to find a broad market as either inhaled or topical formulations.

Synthesis and SAR

The structure of AWD-12-281 differs from other PDE4 inhibitors in that it bears no catechol, but it does feature sub-structures that are common to other PDE4 inhibitors, notably, the 3,5-dichloropyridine that is also present in **roflumilast**. The synthesis of AWD-12-281 is based on modular indole chemistry that allows variation of groups at indole position 1 and the glyoxylate-amides at indole position 3 [**623221**]. In brief, 5-benzyloxyindole is alkylated with 4-fluorobenzyl chloride under basic conditions (potassium hydroxide and dimethylformamide) at the nitrogen to form 5-benzyloxyl-1-(4-fluorobenzyl)indole, which, after precipitation and drying, is reacted with oxalyl chloride to form 3-[5-benzyloxy-1-(4-fluorobenzyl)indolyl]-glyoxyl chloride. The only necessary workup procedure in this step is evaporation. Addition of the glyoxyl chloride to the preformed sodium amide of 4-amino-3,5-dichloropyridine yields 5-benzyl-protected AWD-12-281. After evaporation and two extraction steps, the product crystallizes when concentrated. Finally, the benzyl protecting group is removed by treatment with boron tribromide to give AWD-12-281. The overall yield is 67%, and large-scale yields of 2.85 kg of product have been reported [WO-2004013127].

The originators of AWD-12-281 report that the structure is the result of a 'pharmacophore-based synthesis program' supported by a ligand-based model [**332907**], [**417248**], [**456304**]. In essence, the synthesis described above lends itself to a modular design approach, and thus the interchange of ligand fragments on the basic indole scaffold and a potentially elegant optimization at the process level.

Central to the design concept appears to have been the focus on the catalytic binding site known as the 'low-affinity **rolipram** binding site' (LARBS). One school of thought is that this site is considered less likely to give rise to emetic effects [**304642**]; however, this has not been widely demonstrated to be the case (see below).

A second element in design was the inclusion of a site of metabolism. Unlike **roflumilast** (t1/2 = 7 h) [**412899**], AWD- 12-281 is not metabolized on the dichloropyridine to the N-oxide, rather, the main site of metabolism is on the 5-hydroxy group where glucuronidation takes place. Blockage of this site by a fluorine resulted in AWD-12-343 [WO-03074055], which has an IC50 value of 9 nM against PDE4, and greater oral activity (reduction of lung neurophilia by 90% at 1 mg/kg po) compared with AWD-12-281 (reduction of lung neutrophilia by 37% at 30 mg/kg po) in a rat lipopolysaccharide (LPS)-induced lung neutrophilia model [**372149**], [**383842**]. However, AWD-12-343 has not been advanced into clinical development, perhaps

suggesting that maintenance of a site of metabolism has been an important part of the design of AWD-12-281 and, indeed, this substituent also plays a significant role in the patent protection of the compound.

The follow-up compound for AWD-12-281 is reported to be **ELB-353** (AWD-12-353), an aza-indole that differs from AWD-12-281 only in the indole ring and the absence of the hydroxyl substituent. These variations give rise to an orally available compound that would be likely to have a distinct pattern of metabolism in vivo. The reported activity for **ELB-353** includes high PDE selectivity, high therapeutic index in the ferret and good cardiac tolerance in the pig [**618779**].

Interaction with PDE4 isoforms
The crystal structures of the catalytic domains of PDE4 isoforms with various PDE4 inhibitors (eg, **cilomilast**, **roflumilast** and zardaverine) have been described [**590470**], [**592494**], however, no co-crystal structure of AWD-12-281 has been published. On the basis of data reported for interactions between AWD-12-281 and mutant forms of PDE4, we have developed a summary of the known or likely interactions (described below). The main source of insight into the binding properties of AWD-12-281 comes from a study by Richter et al, in which analysis of amino acid mutations in PDE4 were used to draw conclusions regarding the major points of interaction with inhibitors [**480331**].

Before entering into a detailed description of the reported interactions, it is important to consider some background to these studies, and outline the uncertainties that remain with respect to how PDE4 inhibitors exert desirable pharmacological effects. Firstly, there are a variety of PDE4 splice variants with different properties that are not necessarily conserved between different species, notably rat and human. The splice variants interact with other proteins in the cytoplasm (eg, tyrosyl kinases) via non-catalytic domains, and appear to enter into different states depending on these regulatory interactions [**625446**]. Clearly, conclusions regarding the structural biology of inhibitor binding will depend on the fragment crystallized and the state in which it crystallizes. In general, it appears that crystal structures cover the catalytic domain and are broadly similar across reports [**381233**], [**583746**], [**590470**]. Additionally, distinctions should be made between PDE4 B and D; however, in most crystal structures the main binding determinants in the catalytic site are sufficiently similar that we have generalized across the two in this discussion.

The catalytic domain is considered to represent the so-called LARBS, and most structures show the catechol of **rolipram** interacting with glutamine 369 (PDE4D) or 443 (PDE4B) [**381233**], which is the site of interaction with the purine in AMP. At a functional level, the 'High Affinity **Rolipram** Binding Site' (HARBS) appears to be an allosteric site that is present in intact cells and is associated with neutrophil degranulation effects [**381250**]. By association and correlation, the HARBS is considered to underlie emetic effects in this class; however, this view is still subject to contradictory findings based on new inhibitor data. Rocque et al identified the kinetic basis for differential **rolipram** binding and specifically the requirement for N-terminal sequences 81 to 151 for formation of the HARBS [**381232**]. These observations suggest that structural conclusions on interaction with HARBS cannot be drawn from the crystal data.

Roflumilast as a model
The crystal structures of **cilomilast** and **roflumilast** indicate that there is a conserved interaction at three points in the catalytic site, between the catechol and the glutamine-369 (PDE4D2), between the planar phenyl and the P-clamp and between the terminal pyridine or carboxylate and the metal center [**590470**], [**592494**]. These points of orientation will be used in our interpretation of the data for AWD-12-281.

Interactions with the dichloropyridine group
The dichloropyridine function is considered most likely to interact with the metal binding 'M' pocket. The pyridine N can serve as an H-bond acceptor from the Zn2+ (or Mg2+) bound water. In this configuration there is the potential for the Cl groups to form hydrophobic interactions with nearby amino acids, such as M347, L394 or F414. The dichloropyridine is linked via an amide to the main scaffold and the amide has the potential to interact with bound water in the S-pocket.

Interaction with the P-clamp
The stereotypical interaction between PDE4 inhibitors and the active site of the enzyme is known as the 'P-clamp'. The clamp is based on contributions by amino acids V782, I410, I336, F820 and F446 that line a groove whose state changes with the catalytic state of the enzyme [**480331**]. The planar indole of AWD-12-281 is the most likely point of interaction with the P-clamp

Interactions with the fluorobenzyl group
By structural analogy with **roflumilast**, the terminal fluorobenzyl is likely to interact with the Q2 pocket where hydrophobic interaction is mediated by F414, M411, M431 and F446. Hydrophobic interactions between F645 and the fluorobenzyl are also likely, but potentially very specific, given that F645Y dramatically reduces the sensitivity to AWD-12-281 (F645A is not active) [**480331**]. Hydophobic interactions between F613 and other inhibitors appear important, but are potentially less so for AWD-12-281.

Catechol interactions with glutamine 369
The absence of the catechol oxygens in AWD-12-281 means that the central interaction of known inhibitors is not present in this agent. In the generally accepted model of PDE4 inhibitor binding, the catechol ethers of **roflumilast** or **cilomilast** interact with the P/S pocket on PDE4 [**527734**], where the catechol oxygens form H-bonds with glutamine-369 [**583746**]. It is not clear which substituents, if any, in AWD-12-281 make these interactions.

Interactions with histidine 588
Richter et al demonstrated that mutagenesis of histidine-588 resulted in both a 16-fold reduction in enzyme Ki, and a 24-fold reduction in the IC50 value for AWD-12-281 [**480331**]. This was more profound than for other inhibitors, wherein the reduction was approximately 4- to 9-fold. These higher shifts for AWD-12-281 indicate that the histidine residue is probably the third most effective in moderating AWD-12-281 binding. For **rolipram**, H588 was the fifth most important modification, and for **piclamilast** (**RPR-73401**; an older PDE4 inhibitor no longer in clinical development) it was the fourth [**480331**]. These data do not show a clear role for this histidine residue at the structural level; however, they may show a basis for differential binding by AWD-12-281 and evidence for a less well known binding site for other inhibitors.

AWD-12-281 is reported to be selective between the HARBS and LARBS of PDE4 [**456304**], but a detailed description of the biochemistry of this differentiation is not available. Data regarding selectivity within and between PDE isoforms is derived from enzymes of various species: PDE1 (bovine brain) 11% inhibition at 1 microM; PDE2 (human platelets) 7% at 1 microM; PDE3 (human platelets) 23% at 1 microM; PDE4 (human neutrophils) 50% at 9.7 nM; PDE4 (human recombinant) 50% at 26 nM; PDE5 (human platelets) 20% at 1 microM; PDE6 (bovine retina) 50% at 8 microM; PDE7 (human recombinant) 5% at 1 microM: **rolipram** binding site (human neutrophils) 50% at 0.1 microM [**456304**]. Given the relatively high EC50 for **rolipram** displacement, and the preceding discussions on interactions with mutated forms of the enzyme, it is reasonable to suggest that AWD-12-281 interacts primarily with the LARBS. However, Barnette et al have suggested that HARBS plays a role in neutrophil degranulation [**381250**], and data on this response would be useful in delineating the degree to which the HARBS may be bound by AWD-12-281.

Preclinical Development
The preclinical dataset for AWD-12-281 focuses on its potential use as an inhaled drug. The central argument advanced from the published preclinical data is that the compound has a potentially wide therapeutic window, based on observations made mainly in dog, ferret and pig. These data suggest that potent and long acting effects should be obtained below the emetic threshold in humans.

In vitro
AWD-12-281 is an inhibitor of PDE4 with an IC50 for 'PDE4' (isoform unspecified) activity in human polymorphonuclear leukocytes (PMNLs) of 9.71 +/- 0.51 nM, which is intermediate between **roflumilast** (0.27 +/- 0.02 nM) and **cilomilast** (17.8 +/- 2.32 nM) [**550583**]. These data are broadly comparable with those reported by other researchers in the field, at least for **roflumilast** (with an IC50 value of 0.8 nM against PDE4) [**402539**]; however, in direct enzyme assays against human recombinant PDE4A, the IC50 value of **cilomilast** was recorded at 115 nM with a Ki estimated at approximately 100 nM [**316578**].

AWD-12-281 (1 microM) reduced histamine- and LPS-induced increases in intracellular Ca2+ [**334829**], [**480353**], suggesting that PDE4 inhibitors play a role in Ca2+-induced signal transduction in human monocytes. However, cytokine suppression by AWD-12-281 indicates an anti-inflammatory mode of action as it has been shown to inhibit the release of the pro-inflammatory mediator tumor necrosis factor (TNF)-alpha from human cells involved in allergic inflammatory responses in vitro [**480322**], [**550583**]. The effect of AWD-12-281 on TNF-alpha release from LPS-stimulated whole human blood, LPS-stimulated peripheral blood mononuclear cells (PBMCs) and anti-IgE-stimulated human nasal polyp cells was compared with the effects of **rolipram**, **cilomilast** and **roflumilast** [**480322**], [**550583**] (the concentrations of compound causing half maximum inhibition of cytokine were referred to as EC50 values in [**550583**] and IC50 values in [**480322**]). TNF-alpha release from whole blood was inhibited with EC50 values of 934, 511, 1425 and 17 nM for AWD-12-281, **rolipram**, **cilomilast** and roflunilast, respectively, while from PBMCs, TNF-alpha was inhibited with EC50 values of 87.9, 322.4, 699.7 and 21.2 nM for AWD-12-281, **rolipram**, **cilomilast** and roflunilast, respectively. TNF-alpha release from human nasal polyp cells was inhibited with EC50 values of 111, 156, 190 and 13 nM for AWD-12-281, **rolipram**, **cilomilast** and **roflumilast**, respectively [**550583**], while granulocyte-macrophage colony-stimulating factor (GM-CSF) release from human nasal polyp cells was inhibited with IC50 values of 540, 700 and 500 nM for AWD-12-281, **rolipram** and cilomliast, respectively [**480322**]. PDE4 inhibitors are generally more potent inhibitors of TNF-alpha than GM-CSF, while glucocorticoids tend to be more potent in suppression of GM-CSF than TNF-alpha release [**550586**]. The reduced activity of AWD-12-281 in human whole blood with respect to TNF-alpha suppression could potentially be explained by high plasma protein binding of AWD-12-281 [**550583**].

AWD-12-281 also displayed a broad-spectrum attenuation of various other pro-inflammatory cytokines [**334892**], [**550583**], [**612977**]. In PBMCs co-stimulated with anti-CD3 and anti-CD28 antibodies, a stimulation protocol that gives rise to T-cell proliferation, AWD-12-281 inhibited IL-1beta, IL-2, IL-4, IL-5, IL-8, IL-10, IL-12, IL-13, TNF-alpha and interferon (IFN)-gamma release, with IC50 values of 29, 23, 29, 42, 31, 109, 135, 28, 58 and 84 nM, respectively. The respective IC50 values for these parameters for **roflumilast** were 11, 7 10, 25, 61, 55, 30, 40, 31 and 9 nM, while for **cilomilast** they were 333, 83, 424, 350, not detected, 573, 334, 333, 310 nM and not detected [**612977**]. When compared with either **roflumilast** or **cilomilast** in cytokine suppression, AWD-12-281 is generally intermediate between the two, but for certain parameters, it has notably different potency. These end points include IL-8 and IL-13 inhibition, where AWD-12-281 is more potent than **roflumilast**, and IL-2 and IL-12 (possibly also IFN-gamma), where AWD-12-281 is more similar to **cilomilast** in absolute terms. The differential potency presumably reflects the extent to which PDE4 mediates signaling related to the cytokine and the extent of side activities of the compound. Judging by this end-point alone, AWD-12-281 does not appear to have any unusual side activities. As IL-2, IL-12 and IFN-gamma are considered T-helper 1 (Th1) cytokines, while IL-8 and IL-13 are considered mast cell cytokines (along with TNF-alpha and GM-CSF), suggesting that **roflumilast** is relatively less active on some mast cell cytokines, while **cilomilast** is relatively more active on some Th1 cytokines. These subtle variations in profile may have an impact on the final action and potency of the compounds in vivo and their resultant clinical positioning. With regard to absolute potency, the relatively low effect of AWD-12-281 on release of IL-10 (IC50 = 109 nM) and IL-12 (IC50 = 135 nM) may be the most distinguishing characteristic reported of the in vitro pharmacology.

PDE4 inhibitors have been shown to inhibit macrophage inflammatory protein 1 (MIP1) release from PBMCs and neutrophils [**616643**]. These two cell types were isolated from human blood and pretreated with a PDE4 inhibitor followed by 18 h stimulation with LPS. AWD-12-281, **roflumilast**, **rolipram** and **cilomilast** inhibited MIP1 release from PBMCs with IC50 values of 18.6, 2.12, 206 and 43.1 nM, respectively, while inhibiting MIP1 release from neutrophils with IC50 values of 42.5, 2.21, 134 and 88.2 nM [**616643**].

In vivo
In order to determine efficacy in disease models, AWD-12-281 has been primarily tested in models of airway neutrophilia/eosinophilia or dermatitis [**550581**], [**550586**]. These models are consistent with its current proposed indications of COPD and atopic dermatitis.

Activity in acute airway inflammation
Data have been generated in the rat, ferret and pig using both LPS-induced neutrophilia and allergen-(ovalbumin; ova) induced eosinophilia [**550581**].

AWD-12-281 inhibited allergen-induced late-phase eosinophilia in Brown Norway (BN) rats. In this model, ova challenge in actively ova-sensitized rats leads to eosinophilia in bronchoalveolar lavage fluid (BALF) 48 h after ova challenge. AWD-12-281 (1 to 30 microg/kg) administered intratracheally induced a significant and dose-dependent inhibition of BALF eosinophila in ova-sensitized BN rats when administered 2 h before challenge with ova. The ID50 value for AWD-12-281 was 7 microg/kg intratracheally, while the steroid beclomethasone inhibited eosinophilia by approximately 50% at intratracheal doses of 0.01 to 1 microg/kg [**550581**]. Treatment of BN rats with single intratracheal doses of 30 microg/kg of AWD-12-281 caused a significant reduction of ova-induced increase in eosinophil count in BALF at different times, ranging from -18 to +8 h relative to allergen challenge, by 54 and 35%, respectively. Beclomethase (1 microg/kg administered intratracheally) induced significant inhibition of ova-induced eosinophilia by 46 and 51%, respectively, when administered 6 and 2 h before ova challenge [**550581**].

AWD-12-281 also suppressed allergen-induced early phase bronchoconstriction in sensitized guinea pigs [**550581**], [**578233**]. Strong bronchodilatory effects were observed when AWD-12-281 (1.5 mg/kg) was administered intratracheally 1 h prior to ova challenge, with the ova-induced fall of lung compliace (Cdyn) inhibited by 24% and the rise of lung resistance (RL) inhibited by 68%; however, at the shorter pretreatment time of 30 min, no bronchodilatory or inhibitory effects were observed with oral or intraperitoneal routes of administration of AWD-12-281 at doses up to 30 mg/kg. **Roflumilast** (1.0 mg/kg administered orally) demonstrated a significant inhibition of ova-induced fall in Cdyn by 47 and 33% when administered 1 or 2 h prior to ova-challenge, respectively, with RL inhibited by 85% only at the 2 h time point [**550581**], [**578233**].

In LPS-induced neutrophilia models in the rat, ferret and pig, the ID50 values (inhibition of neutrophilia) for intratracheal administration of AWD-12-281 were 0.1, 10 and 30 µg/kg, respectively; these values correspond to 0.2, 22 and 66 nmol/kg [**550581**], [**612977**]. In contrast, values by other routes were higher, with 1 mg/kg required in the pig intravenously, and 2.4 mg/kg in the ferret intraperitoneally. These data underscore the relatively low systemic availability of AWD-12-281. The corresponding data for **roflumilast** include an EC50 value for rat neutrophilia of 2.7 micromol/kg when delivered orally [**412899**].

The highest doses required to observe an effect were in the pig, wherein 1 mg/kg administered intravenously was approximately equivalent to 0.1 mg/kg administered intratracheally. The emetic dose in pig was in the order of 9 mg/kg when administered intravenously. This provides the basis for an estimated therapeutic index (dose inducing no emesis/ED50) of > 9 by the intravenous route and > 50 by the intratracheal route [**550581**].

Efficacy in dermal models
AWD-12-281 has been characterized in toluene-2,4-diisocyanate (TDI)-induced inflammation of the mouse ear [**480252**], [**550586**]. As observed in the airway models, topical application was the preferred route for AWD-12-281, in this case via an acetone/DMSO formulation. Application of a 0.3% weight per volume solution of AWD-12-281 to an allergen-challenged ear at various intervals after challenge resulted in approximately 75% inhibition of ear swelling. Application of a 3% solution of **cilomilast** led to an approximate 60% reduction in swelling [**480252**]. Application of 3% AWD-12-281 resulted in a near complete suppression of swelling. Administration of AWD-12-281 either orally or intraperitoneally resulted in markedly lower reduction in swelling. AWD-12-281 (30 mg/kg administered orally) had no effect, while **cilomilast** caused an approximately 50% reduction in ear swelling for the same dose and route. Topical application of 3% AWD-12-281 was associated with reductions in levels of IL-4, IL-6 and MIP2 by 50% or more [**550586**].

Metabolism and Pharmacokinetics
AWD-12-281 has a low oral bioavailability of 1 to 5% in rats and 1% in dogs; however, bioavailability in animals reaches 50% after intratracheal administration [**612977**]. After intravenous and oral administration to animals, AWD-12-281 is rapidly cleared from the plasma with a t1/2 of 2 to 3 h and metabolism is primarily via glucuronidation of the indole hydroxy [**612977**]. Application via inhalation results in retention in the lung and slow loss to the systemic circulation. Following intratracheal application in the rat, effect is maintained for over 18 h suggesting that retention in the lung is a significant element in the in vivo pharmacology of AWD-12-281 [**550581**].

In phase I trials, there was no difference between smokers and non-smokers with respect to the pharmacokinetics/bioavailability of AWD-12-281. A bi-exponential plasma level decline was indicative of a sustained release of the compound from the airways [**571646**], [**612977**].

Toxicity
The key toxicity endpoint for AWD-12-281 has been emesis and this has been evaluated in ferrets, pigs and dogs. In systemic application, there were no signs of emesis in ferrets at doses up to 10 mg/kg administered intraperitoneally, but there were initial signs in some animals at 15 or 20 mg/kg. In pigs, 9 mg/kg of AWD-12-281 administered intravenously was the minimal emetic dose, compared with 0.3 mg/kg of intravenous **rolipram**, 0.3 mg/kg of intravenous roflimulast and 9 mg/kg of intravenous **cilomilast** [**550581**]. Emesis was not observed following intratracheal administration of AWD-12-281 in ferrets or pigs at doses up to 10 mg/kg [**612977**]. A dose of 14.05 mg/kg/day administered intratracheally over 4 weeks was tolerated in the dog [**550581**].

In standard rodent toxicological studies, AWD-12-281 administered at doses up to 2.15 g/kg orally or intraperitoneally had no acute effect in rats or mice. Similarly, oral administration to rats (up to 1000 mg/kg) or dogs (up to 215 mg/kg) did not generate clear toxic endpoints. Intranasal applications of AWD-12-281 formulations intended for use in humans were likewise not toxic to rats or dogs over 28 days, albeit at unspecified doses [**456304**]. Damage to rat mucosa after inhalation was not observed [**550581**]. In general, given the relatively low systemic availability of AWD-12-281, these data are not unexpected. Combined with the lack of emesis following high doses by inhalation, they suggest that the compound is likely to be well tolerated in humans, as discussed below.

Clinical Development
Phase I
An inhaled dry powder formulation of AWD-12-281 initially underwent phase I trials with a view to treating asthma and COPD [**457611**], [**571646**], [**612977**]; however, the compound is no longer reported to be in development for asthma [**515870**]. The phase I study indicated that the compound was safe and well tolerated, with doses up to 40 mg/kg/day causing no more adverse events in the individuals receiving AWD-12-281 than in the placebo arm. Furthermore, there was no potentiation of rescue medication-induced cardiac adverse events [**571646**], [**612977**].

GSK and elbion have announced that they are currently conducting phase I trials of a topical cream formulation of AWD-12-281 for the potential treatment of atopic dermatitis, but no study details are available [**582570**].

Phase II
AWD-12-281 was initially considered for phase II trials as an intranasal formulation for allergic rhinitis [**457611**], but announcements by GSK [**515870**] and elbion [**562490**] no longer refer to this indication. It was reported to have progressed to phase II trials as an inhaled formulation for asthma and COPD [**467753**]; however, as stated above, it is no longer in development for asthma [**515870**]. The inhaled formulation is still in phase II trials for COPD.

Side Effects and Contraindications
AWD-12-281 was safe and well tolerated at doses up to 40 mg/kg/day and there was no difference between adverse events in AWD-12-281 and placebo groups [**612977**].

Patent Summary
3-Indolylglyoxylic acids were initially described in 1958 [**624643**], as were 3-indolylglyoxylic acids bearing an oxygen substituent in the 5-position [**623221**]. Given this prior art, the patents covering AWD-12-281 tended to focus on manufacturing routes, processes and pharmaceutical use, rather than on broad chemical structure claims. The hydroxyindole AWD-12-281 appeared first in WO-09955696 (published by **elbion AG** in November 1999), in which the inventors described several methods of how to remove a protective group leading to the liberation of the target molecule AWD-12-281. The synthesis of the precursor molecule is not described but it is published in other places (see above). In ZA-200005540, published in March 2001 (priority number DE-19981018964), the inventors disclosed the formation of the potassium salt of AWD-12-281 and the removal of several different protective groups from the phenolic hydroxyl group. Biological activity was also described. The related application, DE-19917504 published in October 2000 (priority application) gave no examples of chemical synthesis, but described the pharmaceutical use of the compound. More recently, WO-2004006920, published by **elbion AG** in January 2004, claimed the use of AWD-12-281 as a topical agent for treating skin diseases. WO-2004013127, published by **elbion AG** in February 2004, discloses several optimized methods to deprotect the phenolic hydroxyl group and high yielding process methods to provide AWD-12-281 in superior quality. WO-2004022041 published in March 2004 by **elbion AG** claims AWD-12-281 for use in non-allergic rhinitis and chronic sinusitis, although it is not longer in development for these indications. All of these disclosures concentrated on hydroxyindole derivatives, and where the chemistry is described they always included the deprotection of the hydroxyl moiety of the indole as the primary element of novelty. The lead chemist in all of these disclosures appears to have been Norbert Hofgen, who now serves elbion as the Vice President of Chemistry.

There are many other disclosures with varying assignees and inventors that describe uses of AWD-12-281 in combination with other drugs (eg, glucocorticoids and anticholinergic agents). Examples of some of these are: (i) WO-2005013967 (published by **Boehringer Ingelheim GmbH** in February 2005), which claims a combination of an anticholinergic agent and a PDE4 inhibitor, such as **roflumilast** or AWD-12-281, for treating inflammatory or respiratory disorders; (ii) WO-2004019984 (published by **VIATRIS GmbH & Co KG** in March 2004), which claims a combination of a glucocorticoid, such as loteprednol (**VIATRIS GmbH & Co KG**), and at least one PDE4 inhibitor, such as AWD-12-281; (iii) WO-2004004704 (published by **Boehringer Ingelheim GmbH** in January 2004), which claims formulations comprising an anticholinergic agent and at least one PDE4 inhibitor; (iv) WO-2004062671 (published by **ALTANA Pharma AG** in July 2004), which claims PDE4 inhibitors, such as AWD-12-281 alone or in combination with differentiation inducing agents and/or cAMP agonists or stable analogs of cAMP, for treating neoplasm of lymphoid cells;

(vi) WO-2002069945 (published in September 2002 by **Boehringer Ingelheim Pharma KG**), which claims the combination of an anticholinergic agent and a PDE inhibitor, such as AWD-12-281, for the treatment of respiratory diseases, and (vii) WO-03000289 (published in January 2003 by **Glaxo Wellcome plc**; now **GlaxoSmithKline plc**), which claims the treatment of pulmonary diseases, such as COPD or asthma, by administering a PDE4 inhibitor, such as AWD-12-281, in combination with a histamine H1-receptor antagonist.

Current Opinion

The target profile for PDE4 inhibitors originally included their use as systemic replacements for topical steroids in lung diseases, notably asthma and COPD. To date, the clinical performance of PDE4 inhibitors has been marginal. In asthma, both **cilomilast** (15 mg twice daily) and **roflumilast** (0.5 mg once daily) have been shown to provide small benefits in exacerbations and quality of life [**583773**]; however, the effects are limited using the tolerated dose range. Similarly, in a trial of allergen-induced asthma, **roflumilast** (0.25 or 0.5 mg) reduced late allergen responses in a dose-dependent manner, while effects on short-term responses were closer to marginal (in the range of a 25% reduction in asthmatic responses) [**625432**]. The mechanistic basis for this limited response in asthma remains unclear, although it is possible that the compounds are underdosed to avoid systemic side effects. This degree of effect is unlikely to make the compounds compelling replacements for inhaled steroids in asthma.

In COPD, glucocorticoids are less effective and may be more readily substituted for a novel mode of action. Reports of trials of **roflumilast** (250 or 500 microg daily for 24 weeks) in COPD patients indicate that a small, but significant, effect can be obtained via the blockade of PDE4 [**583773**], [**622878**]. At the highest dose reported, there was a significant, but small, increase in post-bronchodilator forced expiratory volume (FEV) of 97 ml and overall health-related quality of life, but 22% of individuals in the high dose group left the study, versus 11% in the placebo arm. Similarly, **cilomilast** (15 mg twice daily) has been shown to provide improvements in lung function in phase III trials [**583773**]. Although these data were sufficient to obtain an approvable letter from the FDA for salbutamol non-responsive COPD [**510678**], this was issued with a concomitant requirement for further efficacy data [**504644**]. This is consistent with the general observation that the extent of response has not fulfilled the more optimistic expectations for PDE4 inhibitors in all trials [**583773**].

The observations in trials and preclinical models suggest that a potent PDE4 inhibitor with limited adverse effects has the potential to deliver some limited improvements in quality of life for COPD sufferers. The question that is to be resolved is whether dosing at higher levels of anti-inflammatory activity will lead to therapeutic benefit. In the **roflumilast** trials noted above, there were signs of dose-responsiveness at the level of FEV, even if absolute response was low. This may suggest that one key to improving outcomes will be increased dose. Data obtained in porcine models and apparent tolerability in initial human studies suggest that there is a good prospect of obtaining a tolerated dose of ADW-12-281 that is adequate to provide significant effect.

There is, as yet, insufficient data to assess prospects for the application of AWD-12-281 in dermal indications. Preclinical data are promising and the in vitro profile for this application is reasonable.

In general, the success of inhaled steroids with high lung retention as therapeutics for chronic asthma is indicative of the potential benefits of controlled drug placement. AWD-12-281 exemplifies the extension of this approach to the PDE4 inhibitors. Delivery in an inhaled form is generally thought to be less attractive than oral formulations; however, for patients experienced with inhaled medicines, this should not be a major factor if inhaled AWD-12-281 is accompanied by good tolerance and clear levels of benefit. The available data suggest that this will be the case and that AWD-12-281 will be competitive versus existing candidates, including **cilomilast**, **roflumilast** and **CC-10004** (**Celgene Corp**).

Biology

Study Type	Effect Studied	Experimental Model	Result	Reference
In vitro	PDE4 inhibition.	PDE4 activity in isolated human PMNLs.	AWD-12-281 inhibited PDE4 activity with an IC50 value of 9.71 +/- 0.51 nM, compared with IC50 values 0.27 +/- 0.02 and 17.8 +/- 2.32 nM for roflumilast and cilomilast, respectively.	**550583**
In vivo	Ear swelling and cytokine production.	TDI-induced model of allergic dermatitis in mice.	Topical application of 3% AWD-12-281 resulted in near complete regression in ear swelling, with associated reductions in levels of IL-4, IL-6 and MIP2 by 50% or more in ear skin.	**550586**
In vivo	Toxicity.	Dogs.	A dose of 14.05 mg/kg/day administered intratracheally for 4 weeks was well tolerated.	**612977**
In vivo	Inhibition of neutrophilia.	LPS-induced neutrophilia in rats, ferrets or pigs.	The EC50 values (inhibition of neutrophilia) for intratracheal administration of AWD-12-281 were 0.1, 10 and 30 microg/kg in rats, ferrets and pigs, respectively.	**550581**
In	Inhibition of	Immunoglobulin E-	TNF-alpha release was inhibited with EC50	**550583**

vitro	TNF-alpha release.	stimulated human nasal polyp cells.	values of 111, 156, 190 and 130 nM for AWD-12-281, rolipram, cilomilast and roflumilast, respectively.

Metabolism

Study Type	Effect Studied	Experimental Model	Result	Reference
In vivo	Bioavailability.	Rats and dogs.	The oral bioavailability of AWD-12-281 was 1 to 5% in rats and 1% in dogs; however, intratracheal bioavailability was 50%.	**612977**
In vivo	Pharmacokinetics.	A phase I study in volunteers receiving up to 40 mg/day of AWD-12-281.	AWD-12-281 displayed low oral bioavailability and rapid clearance from plasma with bi-exponential kinetics indicative of sustained release of compound from the lung.	**612977**

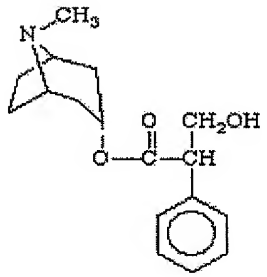
Clinical

Effect Studied	Experimental Model	Result	Reference
Safety and tolerability.	Volunteers receiving up to 40 mg/day of AWD-12-281.	AWD-12-281 was safe and tolerable. Doses up to 40 mg/day could not be distinguished from placebo.	**612977**

TOP

ATTACHMENT B

atropine



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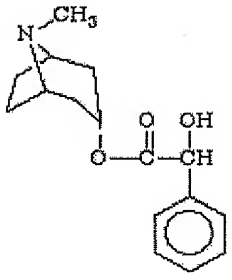
homatrop



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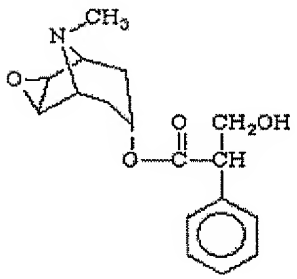
scopolamine



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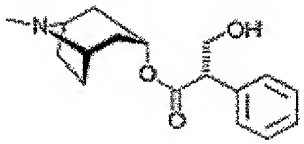
Hyoscyamine

From Wikipedia, the free encyclopedia

Hyoscyamine is a chemical compound, a tropane alkaloid it is the levo-isomer to atropine. It is a secondary metabolite of some plants.

Brand names for hyoscyamine include **Symax**, **Anaspaz**, **Buwecon**, **Cystospaz**, **Levsin**, **Levbid**, **Donnamar**, **NuLev**, and **Neoquess**.

Contents



Hyoscyamine

Systematic (IUPAC) name

(8-methyl-8-azabicyclo[3.2.1]oct-3-yl) 3-hydroxy-2-phenyl-propanoate

Identifiers

CAS number	101-31-5 (http://www.nlm.nih.gov/cgi/mesh/2006/MB_cgi?term=101-31-5&rn=1)
ATC code	A03BA03 (http://www.whocc.no/atcddd/indexdatabase/index.php?query=A03BA03)
PubChem	3661 (http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?cid=3661)
DrugBank	APRD00607 (http://redpoll.pharmacy.ualberta.ca/drugbank/cgi-bin/getCard.cgi?CARD=APRD00607)

Chemical data

Formula	$C_{17}H_{23}NO_3$
Mol. weight	289.375 g/mol

Pharmacokinetic data

Bioavailability	?
Metabolism	?
Half life	?
Excretion	?

Therapeutic considerations

Pregnancy cat.	?
Legal status	?
Routes	Oral, Injection

Uses

It is used to provide symptomatic relief to various gastrointestinal disorders including spasms, peptic ulcers, irritable bowel syndrome, pancreatitis, colic and cystitis. It has also been used to relieve some heart problems and control some of the symptoms of Parkinson's disease. It is anticholinergic, working by inhibting the action of acetylcholine in smooth and cardiac muscle, the sinoatrial and atrioventricular nodes and the exocrine glands.

Side effects

Side effects include eye pain, blurred vision, restlessness, dizziness, arrythmia, flushing, faintness. An overdose will cause headache, nausea, vomiting and CNS symptoms including disorientation, hallucinations, euphoria, inappropriate affect, short-term memory loss and coma.

Isolation

It can be extracted from plants of the *Solanaceae* family, notably *Datura stramonium*. Empirically it is $C_{17}H_{23}NO_3$. Its structural name is α-(hydroxymethyl)-, 8-methyl-8-azabicyclo[3.2.1]oct-3-yl ester, [3(S)-endo]-1αH,5αH-Tropan-3α-ol. It is also known as benzeneacetic acid.

External links

- Link page to external chemical sources.

Retrieved from "http://en.wikipedia.org/wiki/Hyoscyamine"

Categories: Alkaloids | Muscarinic antagonists